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# *NEW WHITE DWARFS IN THE SOUTHERN HEMISPHERE: FOURTH LIST*

By Willem J. Luyten

UNIVERSITY OF MINNESOTA

*Communicated by Harlow Shapley, January 15, 1954*

The northernmost group of proper-motion stars found in the Bruce Proper Motion Survey for which color plates have also been taken with the Bruce telescope is that lying between declinations $-50°$ and $-45°$. Seventy-two pairs of plates were originally blinked in this zone and more than 10,000 stars were marked as possessing appreciable motion. These have been measured and, although the final catalog has not yet been printed, the manuscript has been completed and contains the numbers BPM 30001 through 45375 (stars between $-50°$ and $-40°$ declination). Seventy-two yellow plates were taken with the same telescope and these have now been compared with the original blue plates. Approximate colors have thus been determined in the same manner as before.[1]

The vast majority of these stars are, of course, ordinary red dwarfs, but some 600 were singled out as being less red than expected. Narrowing down the limits still further, 37 stars have been selected as holding out the greatest probability of being genuine white dwarfs. Data for these stars are given in table 1, the several columns of which require no further explanation.

As before, the 37 possible white dwarfs have been arranged in three groups, the first one containing only two stars—namely, those which had been recognized as white on plates taken at the Cordoba Observatory. The second group comprises the 16 stars for which the probability is very high that they are white dwarfs, while the third group contains 19 stars of which some will almost certainly prove not to be

TABLE 1

| BPM | L | R.A. 1950 | DEC. | $m$ | $\mu$ | $p$ | I.C. |
|---|---|---|---|---|---|---|---|
| 31852 | 302-89 | $4^h$ 19.6 | −48° 46′ | 14.8 | 0.″557 | 176° | +0.″4 |
| 37093 | 327-186 | 12 36.1 | −49 33 | 14.0 | 0.556 | 257 | −0.1 |
| 30295 | 291-66A | 0 39.4 | −47 45 | 14.5 | 0.091 | 90 | +0.2 |
| 30551 | 292-41 | 1 04.7 | −46 26 | 15.2 | 0.234 | 70 | +0.1 |
| 31458 | 300-99 | 3 27.9 | −47 48 | 17.0 | 0.177 | 172 | +0.2 |
| 31476 | 300-79 | 3 30.1 | −47 22 | 16.7 | 0.161 | 217 | 0.0 |
| 31594 | 300-34 | 3 41.8 | −45 58 | 15.6 | 0.173 | 33 | +0.1 |
| 33469 | 315-153 | 8 26.8 | −48 09 | 15.6 | 0.098 | 82 | +0.1 |
| 37175 | 327-164 | 12 40.7 | −48 58 | 14.8 | 0.146 | 128 | −0.1 |
| 38165 | 332-123 | 14 07.5 | −47 31 | 14.9 | 0.107 | 268 | 0.0 |
| 39096 | 334-167 | 14 57.7 | −48 39 | 13.2 | 0.101 | 215 | −0.1 |

TABLE 1 (*Continued*)

| BPM | L | R.A. 1950 DEC. | | m | μ | p | I.C. |
|---|---|---|---|---|---|---|---|
| 39332 | 335–172 | $15^h$ $12^m.5$ | −49° 31′ | 14.9 | 0″.105 | 205° | +0$^m$.1 |
| 39481 | 335–31 | 15 22.0 | −46 12 | 14.8 | 0.214 | 244 | +0.1 |
| 40663 | 340–4 | 17 01.8 | −45 10 | 14.4 | 0.122 | 210 | 0.0 |
| 41279 | 342–40 | 17 44.5 | −46 03 | 14.6 | 0.199 | 234 | +0.1 |
| 42361 | 346–51 | 18 48.8 | −46 10 | 14.1 | 0.149 | 163 | +0.1 |
| 44347 | 356–123 | 22 03.9 | −48 34 | 15.5 | 0.117 | 265 | 0.0 |
| 44710 | 358–17 | 22 55.0 | −46 33 | 14.9 | 0.176 | 168 | 0.0 |
| 30559 | 293–95 | 1 05.9 | −48 30 | 14.5 | 0.068 | 168 | +0.2 |
| 31463 | 300–87 | 3 28.2 | −47 40 | 16.0 | 0.070 | 212 | +0.3 |
| 34516 | 319–116 | 9 52.5 | −48 36 | 14.6 | 0.073 | 305 | +0.1 |
| 35137 | 321–102 | 10 26.1 | −47 58 | 14.9 | 0.045 | 242 | +0.1 |
| 35623 | 322–55 | 10 59.3 | −46 42 | 13.0 | 0.126 | 310 | +0.2 |
| 35710 | 323–2 | 11 06.3 | −45 30 | 14.0 | 0.081 | 274 | +0.2 |
| 36430 | 325–214 | 11 53.7 | −48 24 | 12.2 | 0.050 | 277 | 0.0 |
| 38119 | 332–256 | 14 04.6 | −49 51 | 15.2 | 0.076 | 234 | +0.1 |
| 38217 | 332–183 | 14 09.8 | −48 25 | 14.6 | 0.097 | 275 | +0.1 |
| 39158 | 334–185 | 15 01.2 | −48 58 | 14.9 | 0.039 | 210 | 0.0 |
| 39210 | 334–68 | 15 04.0 | −47 00 | 13.4 | 0.095 | 212 | +0.2 |
| 39513 | 335–19 | 15 23.3 | −45 38 | 14.5 | 0.084 | 231 | +0.1 |
| 40663 | 340–125 | 16 46.6 | −48 49 | 15.2 | 0.044 | 119 | 0.0 |
| 40981 | 341–21 | 17 20.2 | −45 26 | 14.8 | 0.035 | 208 | +0.1 |
| 41238 | 342–53 | 17 41.3 | −46 41 | 14.0 | 0.122 | 260 | +0.2 |
| 42769 | 347–43 | 19 19.8 | −46 52 | 14.2 | 0.042 | 166 | 0.0 |
| 43348 | 350–50 | 20 15.7 | −47 16 | 16.6 | 0.037 | 159 | +0.2 |
| 44407 | 356–71 | 22 10.6 | −47 03 | 14.0 | 0.097 | 126 | +0.1 |
| 45042 | 360–96 | 23 33.4 | −48 13 | 14.2 | 0.086 | 114 | +0.1 |

white dwarfs. But while several stars may eventually drop from the list, others from among the 600 are expected to take their place and statistically speaking, the stars in this group should be included with the probable and possible white dwarfs found between declinations −50° and −45°.

If all the stars announced in the present list and in the three previous lists are counted as probable white dwarfs, then the total number of such stars now known has reached 319.

*Acknowledgment.*—It is a pleasure to acknowledge the financial support received from the Rumford Fund of the American Academy of Arts and Sciences which made possible the taking of the yellow plates. The author is further indebted to the Harvard College Observatory, and especially to Dr. Harlow Shapley for arranging to have these plates taken and for his continued interest in and support of this program of research.

[1] These PROCEEDINGS, **37**, 637–640 (1951); **38**, 494–496 (1952); and **39**, 135–137 (1953).

# *RELATION OF POLYPHENOL OXIDASES TO FUNGITOXICITY*

By Saul Rich and James G. Horsfall*

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*Communicated January 14, 1954*

*Stemphylium sarcinaeforme* (Cav.) Wilt. (formerly *Macrosporium sarcinaeforme* Cav.) and *Monilinia fructicola* (Wint.) Honey (formerly *Sclerotinia fructicola* (Wint.) Rehm.) are two fungi which have been widely used as test organisms for screening fungicides in the laboratory. *S. sarcinaeforme* is resistant to the toxicity of phenolic and quinonoid compounds (herein referred to as phenols and quinones) while *M. fructicola* is not. *S. sarcinaeforme* is typically dark colored, while *M. fructicola* is hyaline or buff-colored. As dark pigmentation is frequently associated with polyphenol oxidase activity,[1] it was postulated that *S. sarcinaeforme* must have an active polyphenol oxidase which could oxidize and polymerize phenols and quinones, thus rendering them innocuous to the organism. Polymerization would be an essential part of the detoxification because the mere oxidation of a phenol would probably produce a toxic quinone. It was further postulated that *M. fructicola* probably lacks an active polyphenol oxidase that could detoxify these compounds, or else contains, in vivo, an inhibitor of the system; e.g., an active hydrogen donor.[2] In order to explore these hypotheses, it was first necessary to ascertain what type of polyphenol oxidase, if any, was present in each fungus. The next step was to determine the relationship between fungitoxicity of phenols and quinones and their resistance to being changed by enzymes found in the fungi.

*Methods.*—The fungi were grown in still culture on casamino acid media,[3] *S. sarcinaeforme* for 3 weeks and *M. fructicola* for 2 weeks. Once the mycelial mats were harvested, all succeeding steps for extraction were done at 0° to 5°C. The harvested mats were washed, broken up in a Waring Blendor with 50 cc. of distilled water, and then ground to a cell-free preparation using a tissue homogenizer aided by the addition of powdered alumina. This material was dialyzed overnight and then centrifuged at 18,000 r. p. m. for $^1/_2$ hour. The supernatant was used as the enzyme preparation for the tests to be described.

The activity of the mycelial extract was determined by mixing it with aqueous solutions or suspensions of the phenols or quinones being tested. The basic mixture was 1 cc. of a 1.0% solution or suspension of the test compound, 1 cc. of pH 5.0 buffer and 0.1 cc. of the mycelial extract. A change in color indicated that the test compound was being acted upon by the enzyme(s) present in the extract. Absence of color change indicated that the compound was not oxidized and polymerized.

If polyphenol oxidases were present in the mycelial extracts, failure to give a color reaction with a given compound could result from two causes: (*a*) the compound is not a substrate for the enzyme, or (*b*) the compound is an inhibitor of the enzyme. These two possibilities were distinguished by replacing 1 cc. of the buffer with 1 cc. of a known substrate, catechol, at 0.01 *M* in pH 5.0 buffer. If the catechol in this mixture did not darken, it was concluded that the test compound was an inhibitor of the enzyme. For each compound, four test combinations were

tried. These were (1) test compound plus enzyme, (2) compound plus catechol plus enzyme, (3) compound plus catechol, (4) compound alone. All tubes contained 2.1 cc. of liquid. Whenever one of the ingredients was omitted, an equal volume of pH 5.0 buffer was substituted. Combination 3 was included to see if any darkening in combination 2 might be caused by a reaction between the compound and catechol. Each experiment included appropriate check; i.e., (*a*) 0.1 enzyme plus 2 cc. of pH 5.0 buffer, (*b*) 1 cc. of 0.01 *M* catechol plus 1.1 cc. of buffer, and (*c*) 1 cc. of 0.01 *M* catechol plus 0.1 cc. of enzyme plus 1 cc. of buffer.

The fungitoxicity data were obtained using the depression slide technique described by Horsfall and Rich.[4] A compound is considered to be toxic if its ED 50 is 50 $\mu$g. per square centimeter or less.

*Experimental Results.*—Both fungi were found to contain polyphenol oxidases. The mycelial extract from *S. sarcinaeforme* contained a polyphenol oxidase capable of acting upon hydroquinone and catechol but not tyrosine. The extract from *M. fructicola* gave color reactions with tyrosine and catechol but not with hydroquinone. This indicated that the polyphenol oxidase for *S. sarcinaeforme* was of the laccase type, while that from *M. fructicola* was of the tyrosinase type.[5]

Table 1 includes the data on the fungitoxicity of 42 phenols and quinones† and their interactions with the extracts from the two fungi. Twenty-three of these compounds were toxic to *S. sarcinaeforme*, and 34 were toxic to *M. fructicola*.

With the mycelial extract of *S. sarcinaeforme*, 17 of the test compounds gave color reactions, and 25 did not. Fifteen of the compounds which could be changed by the enzyme were non-toxic to the spores of the fungus, and only 2 were toxic. On the other hand, 21 of those which gave no color reaction with the mycelial extract of *S. sarcinaeforme* were toxic to its spores, and 4 were non-toxic. Of the 21 compounds which gave no color change with the mycelial extract, six were actually inhibitors of the action of the enzyme on catechol and all six were toxic to the spores. These inhibitors were $\alpha$-naphthol, $\beta$-naphthol, 2,4-dihydroxyterephthalic acid, 3,5-diiodotyrosine, hexyl resorcinol, and 2-chloro-4,6-dinitrophenol. Certain of the compounds were tested to see whether they would inhibit the action of the extract on hydroquinone. Three compounds, all toxic, were found to inhibit this reaction even though they did not inhibit the reaction with catechol. These compounds were 2,5-bis (2,3,4,6-tetramethylphenyl) hydroquinone, cyclohexylhydroquinone, and protocatechuic acid.

Only seven of the compounds gave color reactions with the mycelial extract of *M. fructicola*. Of these seven, two were toxic to the spores of *M. fructicola* and five were non-toxic. Of the 35 compounds not giving color reactions, 32 were toxic and 3 were non-toxic. Three of these 35 compounds were also inhibitors of the action of the extract on catechol. These three were cyclohexylhydroquinone, hexylresorcinol, and the sodium salt of gentisic acid, but only the first two were toxic.

*Discussion.*—Inhibition of vital enzyme systems has been proposed many times as a mechanism of fungitoxicity.[6] The phenols and quinones have been shown by Owens[7] to be inhibitors of pancreatic and malt amylase. Owens suggested the inhibition of amylases as a possible mechanism for the fungitoxicity of these compounds. In a previous paper, Owens[6] tested the activity of a polyphenol oxidase from *Agaricus campestris* L. on a series of phenols and quinones. He found that the enzyme could oxidize phenol but could not oxidize hydroquinone or the quinones

tested. Owens suggested that phenol, hydroquinones, *p*-benzoquinones and 1,4-naphthoquinone were fungitoxic because they might be competitive inhibitors of a polyphenol oxidase. In the present studies, however, hydroquinone, 1,2-benzoquinone, tetrachloro-*p*-benzoquinone, and 2,3-dichloro-1,4-naphthoquinone did not inhibit the polyphenol oxidases of *S. sarcinaeforme* and *M. fructicola*.

The present data for the extract of *S. sarcinaeforme* (table 1), indicate that if the oxidase present in the fungus can oxidize and polymerize the phenols or quinones, the compound is usually non-toxic to the fungus. Of the compounds giving color with the fungus extract, 88% were non-toxic to the spores of the fungus. Polyphenol oxidase then, may act as a detoxifying agent. The only toxic compounds which gave color with the extract of *S. sarcinaeforme* were 4-phenylcatechol and *o*-quinonedioxime. The toxicity of these two compounds indicates (*a*) their toxicity is not directly related to their phenolic or quinonoid structure, e.g., it is known that *o*-quinonedioxime can form metal chelates, which has been proposed as a fungitoxic mechanism,[8] or, (*b*) the polyphenol oxidase of the spore is different from that of the mycelium. This latter possibility will be discussed later. Of the compounds which could not be visibly changed by the extract, 84% were toxic to the fungus. Six of these were inhibitors of laccase-catechol and three were inhibitors of laccase-hydroquinone. The group of 15 non-inhibitors contained 11 fungitoxicants. This indicates that the fungitoxicity of such compounds may be related to some property other than their inhibition of polyphenol oxidase. When the polyphenol oxidase system of *S. sarcinaeforme* is inhibited, however, the compounds doing so were all fungitoxic without exception. Apparently polyphenol oxidase is an important enzyme in the life process of this fungus, probably acting as a terminal oxidase.

Four of the compounds were not toxic to the spores of *S. sarcinaeforme*, even though they gave no color reaction with the mycelial extract. These four were *p*-cyclohexylphenol, *p*-nonylphenol, 2-capryl-4,6-dinitrophenol and *l*-tyrosine. These compounds are all monophenols with a large aliphatic substituent. It is entirely possible that the aliphatic groups are too large for good permeation into the spores of *S. sarcinaeforme*. The relation of aliphatic groups to permeation and fungitoxicity has been discussed by Rich and Horsfall.[9] They point out that the constitution of the semipermeable membranes of the spores of various fungi may vary considerably, and that this in turn may result in a compound being able to permeate easily into the spores of one fungus and with difficulty into the spores of another. Further, Horsfall[10] has indicated that, for toxicity, *M. fructicola* requires compounds with larger lipophilic groups than does *S. sarcinaeforme*. This is supported by the fact that the *p*-cyclohexyphenol, *p*-nonylphenol, and 2-capry-4,6-dinitrophenol are all toxic to *M. fructicola*. *l*-Tyrosine, the only one of the four not toxic to *M. fructicola*, is a substrate for its mycelial extract.

Of the test compounds which could not be changed by the mycelial extract from *M. fructicola*, 92% were toxic to the spores of this fungus. The exceptions were 4-amino-2-phenylphenol, *o*-nitrophenol, and the sodium salt of gentisic acid. These may be non-toxic because they simply do not penetrate into the spore to the site of toxic action. This would be most likely in the case of the sodium salt of gentisic acid, as highly ionized organic compounds do not penetrate semipermeable membranes very well.

TABLE 1

INTERACTIONS OF PHENOLS AND QUINONES WITH MYCELIAL EXTRACTS OF *S. sarcinaeforme* AND *M. fructicola;* AND THE TOXICITY OF THESE COMPOUNDS TO THE SPORES OF THE TWO FUNGI

| COMPOUND | WITH *S. sarcinaeforme* EXTRACT | | | WITH *M. fructicola* EXTRACT | | |
|---|---|---|---|---|---|---|
| | COLOR CHANGE | INHIBITION OF CATECHOL COLORATION | TOXICITY TO SPORES OF *S. sarcinaeforme* | COLOR CHANGE | INHIBITION OF CATECHOL COLORATION | TOXICITY TO SPORES OF *M. fructicola* |
| 4-Amino-2-phenylphenol | + | − | − | − | − | − |
| Bis(5-*tert* butyl-2-hydroxyphenyl) sulfide | + | − | − | − | − | + |
| Catechol | + | − | − | + | − | − |
| 2,4-Diamylphenol | + | − | − | − | − | + |
| 2′,5-Dihydroxybenzenesulfonamide | + | − | − | − | − | + |
| Gentisic acid, sodium salt | + | − | − | − | + | − |
| Hydroquinone | + | − | − | − | − | + |
| *p*-Isooctylphenol | + | − | − | − | − | + |
| *o*-Nitrophenol | + | − | − | − | − | − |
| 1-Phenanthrol | + | − | − | − | − | + |
| 9-Phenanthrol | + | − | − | − | − | + |
| 2-Phenylamine(N)sulfonylhydroquinone | + | − | − | − | − | + |
| 1,1,3,3-Tetramethylbutylhydroquinone | + | − | − | − | − | + |
| 1,3,5-Trimethylaminophenol | + | − | − | − | − | + |
| Propyl gallate | + | − | − | + | − | − |
| 4-Phenylcatechol | + | − | + | + | − | + |
| *o*-Quinonedioxime | + | − | + | − | − | + |
| 1,2-Benzoquinonedioxime | − | − | + | − | − | + |
| 2,5-Bis(2,3,4,6-tetramethylphenyl) hydroquinone | − | −[a] | + | − | − | + |
| Protocatechuic acid | − | −[a] | + | + | − | − |
| Cyclohexylhydroquinone | − | −[a] | + | − | + | + |
| 2-Chloro-3,6-dinitrophenol | − | + | + | − | − | + |
| 2,5-Dihydroxyterephthalic acid | − | + | + | − | − | + |
| 3,5-Diiodotyrosine | − | + | + | − | − | + |
| Hexylresorcinol | − | + | + | − | + | + |
| α-Naphthol | − | + | + | + | − | + |
| β-Naphthol | − | + | + | − | − | + |
| Chloranil | − | − | + | − | − | + |
| 2,3-Dichloro-1,4-naphthoquinone | − | − | + | − | − | + |
| 4-Chloro-3-phenylphenol | − | − | + | − | − | + |
| *p*-Nitrophenol | − | − | + | − | − | + |
| 2-Phenanthrol | − | − | + | − | − | + |
| 3-Phenanthrol | − | − | + | − | − | + |
| 4-Phenanthrol | − | − | + | − | − | + |
| *o*-Phenylenediamine | − | − | + | + | − | − |
| *p*-Phenylenediamine | − | − | + | − | − | + |
| Salicylanilide | − | − | + | − | − | + |
| Salicylic acid | − | − | + | − | − | + |
| 2-Capryl-4,6-dinitrophenol | − | − | − | − | − | + |
| *p*-Cyclohexylphenol | − | − | − | − | − | + |
| *p*-Nonylphenol | − | − | − | − | − | + |
| *l*-Tyrosine | − | − | − | + | − | − |

[a] Inhibition of hydroquinone coloration with *S. sarcinaeforme* extract.

Of the seven compounds which could be changed by the extract, only two, or 29%, were toxic. These two, 4-phenylcatechol and $\alpha$-napthol, indicate a fundamental difference between the physiology of mycelium and spores. The possibility of a difference in physiology was suggested by Duggar[11] as early as 1901. Recently, Horsfall, *et al.*,[12] have emphasized this difference in discussing discrepancies between fungicide screening techniques using spore germination as a standard and those using mycelial growth.

Under the microscope, the germ tubes of *M. fructicola* spores take on a dark color when exposed to sublethal doses of $\alpha$-naphthol and 4-phenylcatechol. The spores, however, remain hyaline. The toxicity of these two compounds indicate that they permeate the spores. These compounds, however, cannot be changed by the polyphenol oxidase of the spores, but are oxidized and polymerized by the polyphenol oxidase of the mycelium and germ tubes. Although catechol, *o*-phenylenediamine, protocatechuic acid, propyl gallate, and *l*-tyrosine are all acted upon by the mycelial extract of *M. fructicola* and are non-toxic to the spores, only the first three darken both the germ tubes and spores. This indicates that these three compounds permeate the spores and can be changed by the polyphenol oxidases present in both spores and mycelium. Propyl gallate and *l*-tyrosine, however, probably do not permeate either the spores or germ tubes of this fungus. *o*-Nitrophenol, which does not give a color reaction with the mycelial extract of *M. fructicola* and is non-toxic to its spores, colored neither spores nor germ tubes. Observations of this type cannot be made on *S. sarcinaeforme* because the natural dark color of the spores would mask any subsequent color changes.

The susceptibility of *M. fructicola* spores to phenols and quinones is not related, as originally proposed to the complete absence of a detoxifying polyphenol oxidase. Rather, the susceptibility appears to be the result of the restricted substrate range of the polyphenol oxidase present. The mycelial extract of *M. fructicola* could oxidize and polymerize only 17% of the compounds tested, while that of *S. sarcinaeforme* could change 41%. The induction of coloration of the spores and germ tubes of *M. fructicola* by catechol indicates an active polyphenol oxidase in vivo. The normal light color of the fungus, therefore, cannot be caused by either a very low concentration of enzyme, or the presence of an active hydrogen donor which would keep the reaction from going to completion. The light color is probably caused by a very low concentration of natural substrate.

There were three inhibitors of the action of *M. fructicola* extract on catechol. These inhibitors were cyclohexylhydroquinone, hexylresorcinol, and the sodium salt of gentisic acid. Only cyclohexylhydroquinone and hexylresorcinol were toxic to the spores of the fungus. It is interesting that these two compounds can inhibit the polyphenol oxidases from both fungi. A reason for the non-toxicity of sodium gentisate has already been discussed.

Protocatechuic acid is of particular interest in these studies because of its suggested relation to the natural resistance of onions to onion smudge.[13] Protocatechuic acid has been reported to be oxidized by tyrosinase,[5] and is indeed oxidized and polymerized by the enzyme of *M. fructicola* to which it is non-toxic. Protocatechuic acid, however, is an inhibitor of the reaction between hydroquinone and the polyphenol oxidase from *S. sarcinaeforme* and is toxic to the spores of this fungus.

Enzymes, then, vary in their specificity according to the source of the preparation. It seems worth while, therefore, to use enzymes made from the specific fungi involved when making studies relating enzyme inhibition to fungitoxicity. It is also obvious that the results of such studies will vary not only with the enzyme, but also with the substrate. Ideally, the fungus enzyme and its naturally occurring substrate should be used. At any rate, the test substrate should approximate the natural substrate in its affinity for the enzyme. This is particularly true if enzyme inhibition is to be used as an in vitro technique for screening fungicides. A technique of this nature would be very valuable in screening for compounds to be used for preventing the deterioration of wood, fabrics, paint, plastics, etc. Many of the fungi which are particularly destructive to these materials are very poor spore formers in culture. Hence the standard spore germination techniques cannot be used. Enzyme-inhibition is also advantageous as a fungitoxic mechanism in these cases because toxicity to the host is unimportant.

*Summary.*—Forty-two phenols and quinones were tested as fungitoxicants on the spores of *S. sarcinaeforme* and *M. fructicola*. In turn, mycelial extracts of the two fungi were tested for their ability to oxidize and polymerize the same 42 compounds.

The extract of *S. sarcinaeforme* contained a polyphenol oxidase of the laccase type; i.e., giving color reactions with hydroquinone and catechol but not with tyrosine. This extract could oxidize and polymerize 41% of the test compounds. The extract from *M. fructicola* contained a polyphenol oxidase of the tyrosinase type; i.e., oxidizing and polymerizing tyrosine and catechol but not hydroquinone. This extract gave color reactions with only 17% of the test compounds.

The 42 compounds were tested as inhibitors of the two polyphenol oxidases, using catechol as the substrate. Certain of the compounds were tested as inhibitors of the laccase-hydroquinone system.

All but two of the compounds which gave color reactions with extract from *S. sarcinaeforme* were non-toxic to the spores of the fungus. Almost all of the compounds which were not visibly changed were toxic. Many of the latter compounds were found to be inhibitors of the polyphenol oxidase from *S. sarcinaeforme*. All of these enzyme inhibitors, without exception, were toxic to the spores of the fungus.

The correlation between the interaction of the mycelial extracts with test compounds and the toxicity of these compounds to spores was poorer for *M. fructicola* than it was for *S. sarcinaeforme*. The discrepancies, however, could be explained on the basis of (*a*) differences in permeation, and (*b*) differences in substrate specificity between the polyphenol oxidases of the mycelium and that of the spores. These differences were substantiated by observing the presence or absence of color changes in the spores and germ tubes of *M. fructicola* spores which had germinated in sublethal concentrations of the test compounds.

It is postulated that the polyphenol oxidases of *S. sarcinaeforme* and *M. fructicola* can detoxify certain phenols and quinones. *M. fructicola* appears to be the more susceptible of the two fungi to being poisoned by these compounds. This may be caused by the more limited substrate range of the polyphenol oxidases in *M. fructicola*.

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† The majority of these compounds were furnished by the Rohm and Haas Co., Philadelphia, Pa., the Chemical-Biological Coordination Center, N.R.C., Washington, D. C., or purchased from Distillation Products Industries, Eastman Organic Chemicals Department, Rochester, N. Y.

[1] Bonner, J., *Plant Biochemistry*, Acad. Press, New York City, 1950, pp. 181–182.
[2] Rich, S., and Horsfall, J. G., *Phytopathology*, **42**, 473 (1952).
[3] Dimond, A. E., *et al.*, *Conn. Agr. Expt. Sta. Bull.* 531 (1949).
[4] Horsfall, J. G., and Rich, S., *Contrib. Boyce-Thompson Inst.*, **16**, 313–347 (1951).
[5] Burris, R. H., in Lardy (Ed.), *Respiratory Enzymes*, Burgess Publishing Co., 1949.
[6] Owens, R. G., *Contrib. Boyce-Thompson Inst.*, **17**, 221–263 (1953).
[7] Owens, R. G., *Ibid.*, **17**, 273–282 (1953).
[8] Zentmyer, G. A., *Science*, **100**, 294–295 (1944).
[9] Rich, S., and Horsfall, J. G., *Phytopathology*, **42**, 457–460 (1952).
[10] Horsfall, J. G., *Ibid.*, **41**, 658 (1951).
[11] Duggar, B. M., *Bot. Gaz.*, **31**, 38–66 (1901).
[12] Horsfall, J. G., *et al.*, *Natl. Res. Council Publ.* **206**, 210–242 (1950).
[13] Link, K. P., *et al.*, *J. Biol. Chem.*, **81**, 369–375 (1929).

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## *A REFINEMENT OF THE PAULING THEORY OF FERROMAGNETISM*

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In his paper on ferromagnetism[1] Pauling suggested that his theory might be refined by a consideration of the non-uniform probability distribution of electrons in crystals of ferromagnetic substances. In the simple theory it is assumed that the conduction electrons spend equal amounts of time in all parts of the crystal. This is a bad assumption, because a conduction electron with a given direction of spin spends a greater amount of time in regions where its spin is aligned parallel with the spin of the neighboring atomic electrons than in regions where the spin is aligned antiparallel. This paper describes a method of modifying the Pauling theory to take into account the non-uniform electron density.

One of the major advantages of the simple theory of ferromagnetism is the ease of calculation of the partition function for the crystal. Since the electron density is assumed to be uniform each atomic moment may be considered to be interacting with the internal field independently of the other atomic moments. The partition function is therefore of the form

$$f = \left( \sum_{s_z=-1}^{s_z=+1} e^{-\frac{M_V Q}{kT} s_z} \right)^{N_T}$$

where $M_V$ is the net magnetic moment per atom caused by the uncoupling of valence electrons, $Q$ is the interaction energy of a valence electron with the two atomic $3d$ electrons, $S_z$ is the $z$ component of spin of the atomic electrons in a given atom, and $N_T$ is the number of atoms in the crystal.

If the idea of uniform electron distribution is abandoned this simple form for the partition function must, strictly speaking, also be abandoned. The density of conduction electrons in a given small region of the crystal depends upon the degree of alignment of the atomic spins in that region. The extent of further alignment of atomic spins depends upon the density of conduction electrons. Consequently, further alignment of atomic spins will tend to occur in regions where

the existing amount of alignment is greater. The atomic spins are no longer independent, and the partition function must describe the crystal as one large system, rather than as $N_T$ independent ones.

In order to make the calculation simpler it is assumed that a single value of $M_V$ is sufficient to describe the energy of the entire crystal. The quantity $M_V$ takes into account the added uncoupling effect of the unequal distribution of valence electrons, but averages this effect over the entire crystal. The partition function has the same form under these restrictions as it does in the simple theory.

*Calculation of $M_V$.*—The case in which the atomic moment is 1 will be considered first. It is assumed that the probability of finding a conduction electron with spin $+1/2$ in the neighborhood of a given atom is $R_1$ times greater if the atom has atomic spin component $S_z = 0$ than if it has $S_z = -1$, and $R_2$ times greater if the atom has $S_z = +1$ than if it has $S_z = -1$.

If the number of atoms with $S_z = 1$ is called $N_U$, the number with $S_z = 0$ is called $N_0$, and the number with $S_z = -1$ is called $N_D$, then the conduction electrons with spin $+1/2$ are stabilized by an amount of energy proportional to

$$\frac{R_2N_U - N_D}{R_2N_U + R_1N_0 + N_D}.$$

Electrons with spin $-1/2$ have a greater probability density in the neighborhood of atoms with $S_z = -1$ and a smaller probability density in the neighborhood of atoms with $S_z = +1$. The value of $M_V$ is assumed to be proportional to the difference between the two oppositely displaced energy curves[2] for the conduction electrons of spin $+1/2$ and $-1/2$.

$$M_V = \frac{\alpha}{2}\left\{\frac{R_2N_U - N_D}{R_2N_U + R_1N_0 + N_D} + \frac{N_U - R_2N_D}{N_U + R_1N_0 + R_2N_D}\right\}. \qquad (1)$$

The expression is symmetric with respect to interchange of $N_U$ and $N_D$. It reduces to the simple form of the earlier theory when $R_1 = R_2 = 1$. $M_V$ is then proportional to $M_S$, the net atomic spin component per atom in the $z$ direction:

$$M_S \equiv \frac{2(N_U - N_D)}{N_T}; \qquad M_V = \frac{\alpha}{2}M_S$$

when $R_1 = R_2 = 1$.

The partition function provides one relationship between $M_V$ and $M_S$. A second expression is necessary in order to fix $M_V$ and $M_S$ as a function of temperature. The quantity $M_S$ is a single-valued function of $M_V$ in the simpler theory. If equation (1) is restated in terms of $M_S$, it is found that this is no longer true. Instead, the equation is of the form

$$M_V = A(N_0)M_S^2 + B(N_0)M_S + C(N_0). \qquad (2)$$

The coefficients $A$, $B$, and $C$ are functions of $N_0$, and $N_0$ may vary over a wide range of values without affecting $M_V$.

The solution of equation (2) for $M_S$ may be written in the form of a power series in $N_0$, when $M_V$ is small:

$$M_S \cong \frac{M_V}{N_T^4}\left[C_1N_T^4 + C_2N_T^3N_0 + C_3N_T^2N_0^2 + C_4N_TN_0^3 + C_5N_0^4\right]. \qquad (3)$$

In order to provide a single-valued relationship between $M_V$ and $M_S$, an average value of $M_S$ must be calculated for each value of $M_V$. When the power series expansion for $M_S$ is valid we have

$$\bar{M}_S = \frac{M_V}{N_T{}^4}\left[C_1N_T{}^4 + C_2N_T{}^3\overline{N_0} + C_3N_T{}^2\overline{N_0{}^2} + C_4N_T\overline{N_0{}^3} + C_5\overline{N_0{}^4}\right]$$

The Curie temperature may then be found by relating the limiting slope of the Brillouin function to $d\bar{M}_S/dM_V$ evaluated at $M_V = 0$:

$$\left[\frac{d\bar{M}_S}{dM_V}\right]_{M_V=0} = \frac{1}{N_T{}^4}\left[C_1N_T{}^4 + C_2N_T{}^3\overline{N_0} + C_3N_T{}^2\overline{N_0{}^2} + C_4N_T\overline{N_0{}^3} + C_5\overline{N_0{}^4}\right]_{M_V=0}.$$

In order to evaluate this derivative it is necessary to determine the average values of the powers of $N_0$ at $M_V = 0$. In iron all values of $N_0$ from 0 to $N_T$ are consistent with $M_V = M_S = 0$. The only restriction is that $N_U - N_D = 0$. Since the energy of all states with $M_V = 0$ is the same it is sufficient to consider the degeneracy associated with a given value of $N_0$. The degeneracy is given by

$$\omega(N_0) = \frac{N_T!}{(N_T!)\left(\dfrac{N_T - N_0}{2}!\right)^2}.$$

Therefore we obtain

$$\bar{N}_0 = \frac{\sum_{N_0=0}^{N_T} N_0\omega(N_0)}{\sum_{N_0=0}^{N_T}\omega(N_0)}.$$

It is found that $\omega$ has its maximum value at $N_0 = N_T/3$. The maximum is so sharp that a negligible error is made if it is assumed that $\overline{N_0{}^n} = (N_T/3)^n$, where $n$ is equal to 1, 2, 3, or 4, the only powers retained in the power series expansion given in equation (3).

The method of analysis is the same for nickel as for iron, except that the minimum value of $N_0$ is 0.715 $N_T$. The quantity $\omega$ falls off extremely rapidly in the region from 0.715 $N_T$ to $N_T$, and it is therefore found that $\overline{N_0{}^n} = (0.715\ N_T)^n$, for small values of $n$.

In the case of cobalt 28.5% of the atoms have atomic spin $^3/_2$ and 71.5% have atomic spin $^1/_2$. The atoms with spin $^3/_2$ may have spin component $+^3/_2$, $+^1/_2$, $-^1/_2$, or $-^3/_2$. Atoms with spin $^1/_2$ may have spin component $+^1/_2$ or $-^1/_2$. The value of $M_V$ is given by

$$M_V = \frac{\alpha_{Co}}{2}\left\{\frac{^3/_2R_{3/2}N_{3/2} + {}^1/_2R_{1/2}N_{1/2} - {}^1/_2R_{-1/2}N_{-1/2} - {}^3/_2N_{-3/2}}{R_{3/2}N_{3/2} + R_{1/2}N_{1/2} + R_{-1/2}N_{-1/2} + N_{-3/2}} + \right.$$
$$\left.\frac{^3/_2N_{3/2} + {}^1/_2R_{-1/2}N_{1/2} - {}^1/_2R_{1/2}N_{-1/2} - {}^3/_2R_{3/2}N_{-3/2}}{N_{3/2} + R_{-1/2}N_{1/2} + R_{1/2}N_{-1/2} + R_{3/2}N_{-3/2}}\right\}$$

where the number of atoms with spin component $i$ is $N_i$, and the factor of increased probability of finding a conduction electron of spin $+^1/_2$ near an atom with spin component $i$ is $R_i$. In the case of cobalt we use the definition

$$M_S \equiv \frac{{}^3/_2N_{3/2} + {}^1/_2N_{1/2} - {}^1/_2N_{-1/2} - {}^3/_2N_{-3/2}}{N_{3/2} + N_{1/2} + N_{-1/2} + N_{-3/2}}.$$

The procedure for finding a relationship between $M_S$ and $M_V$ for cobalt is like that for iron and nickel, except that in finding the average $M_S$ it is necessary to sum over two parameters, $N_{1/2}$ and $N_{-1/2}$.

It is possible to find values for the Curie temperatures of iron, cobalt, and nickel, provided that the values of the $R_i$ for the three elements are known. In order to demonstrate roughly the nature of the corrections provided by this refinement it may be assumed that the values of $R_1$ and $R_2$ are the same for iron as for nickel, and that $R_2$ is equal to $R_1^2$. The possible change in the predicted Curie temperature of nickel, shown in table 1, is not very great if one makes this assumption, but every choice of $R_1$ results in a decrease in the predicted Curie temperature of iron and an increase in that of nickel. This has also been found to be true over a very wide range of the parameters $R_1$ representing considerable deviations from the restriction $R_2 = R_1^2$.

TABLE 1

| $R_1$ | $T_C$(Fe) | $T_C$(Ni) |
|---|---|---|
| 1.00 | 1395°K.[a] | 370°K.[a] |
| 1.50 | 1281 | 374 |
| 1.81 | 1168 | 377 |
| 2.00 | 1100 | 378 |
| 2.16 | 1045 | 379 |
| 4.00 | 630 | 383 |

[a] The values given as the result of the simple theory differ from the published values, because those given here have been corrected to take account of the thermal expansion of the metal. It has been shown that thermal expansion is the only temperature effect of importance in determining the positions of the energy levels. The thermal uncoupling effect, which is of importance in explaining temperature-independent paramagnetism, makes a negligible contribution in this case (work of P. Schatz and G. Felsenfeld, unpublished).

An examination of equation (1) shows that one effect of making $R_2$ greater than 1 is to decrease the uncoupling of valence electrons. This is the predominant effect in iron, and the Curie temperature decreases. In nickel, however, the presence of large numbers of atoms with $S_z = 0$ tends to concentrate the valence electrons in the region where uncoupling is possible when $R_2$ is greater than $R_1$. In such a case, increasing $R_2$ will increase uncoupling, and the Curie temperature will be raised.

An attempt has been made to determine the relationship among the parameters $R_i$ more accurately, using a periodic square-well potential as a model of the metal crystal. The differences between iron and nickel in the depth and width of the wells may be taken into account. It is also possible to estimate the previously neglected dependence of the parameters $R_i$ upon the numbers $N_U$, $N_0$, and $N_D$. This study indicates that although the restriction $R_2 = R_1^2$ is a reasonable one for iron, $R_2$ should be somewhat greater than $R_1^2$ in the case of nickel. The analysis provides a relationship among the parameters $R_i$ which will cause a predicted increase of about 100° in the Curie temperature of nickel when the decrease in iron is chosen to give the experimentally observed Curie temperature. In these cases, the predicted Curie temperature of cobalt remains virtually unchanged, as might be expected from its intermediate position and structure.

*Conclusion.*—The Pauling theory of ferromagnetism has been modified by introducing an assumption of non-uniform electron distribution in the ferromagnetic crystal. An expression for the Curie temperature is derived, assuming that the effects of the non-uniformity may be averaged over the entire crystal. If reasonable sets of the numbers $R_i$ which describe the non-uniformity are inserted in this expression, the result is an increase in the predicted Curie temperature of nickel and a decrease in that of iron, both of which are improvements.

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[1] Pauling, L., PROC. NATL. ACAD. SCI., **39**, 551 (1953).

[2] Seitz, F., *The Modern Theory of Solids*, McGraw-Hill, New York, 1940, p. 159.

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## *ON MOST PROBABLE DISTRIBUTIONS*

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*1. Introduction.*—In modern discussions of techniques in statistical mechanics it is rightly emphasized[1] that one is interested in mean occupation numbers of states as averaged over ensembles of systems (of particles). Nonetheless the older method of searching for the most probable occupation numbers of the states in a system of interest, *without reference to ensembles*, is still widely used, either as a guide to what one may expect for the mean occupation number, or as a means of deriving new results. The present object is to analyze this method somewhat more carefully than appears to have been done so far in textbooks or papers.[1–4] As conventionally presented the method is subject to the criticism that it involves Stirling's approximation, which is valid only if certain integers (including the most probable occupation numbers which one is trying to determine by this method) are large. We shall show (i) that this assumption can be avoided. The crucial step in the argument is then seen to occur when one ignores the fact that certain numbers are integers, and treats them instead as if they could vary continuously. We shall call this "the assumption of continuity," and show (ii) that this assumption can rule out what are in fact the most probable distributions.

*2. The Elimination of Stirling's Approximation.*—We shall consider[3] the distribution of $N$ independent particles over levels having energies $E_1, E_2, \ldots$ and degeneracies $\omega_1, \omega_2, \ldots$. Write $G = G_1G_2\ldots$ for the number of ways of realizing a given state $(n_1, n_2, \ldots)$ of the whole system, which is specified by the numbers $n_1, n_2, \ldots$ of particles in the energy levels $E_1, E_2, \ldots$ respectively. Apart from constant factors, which do not interest us here, one has[3]

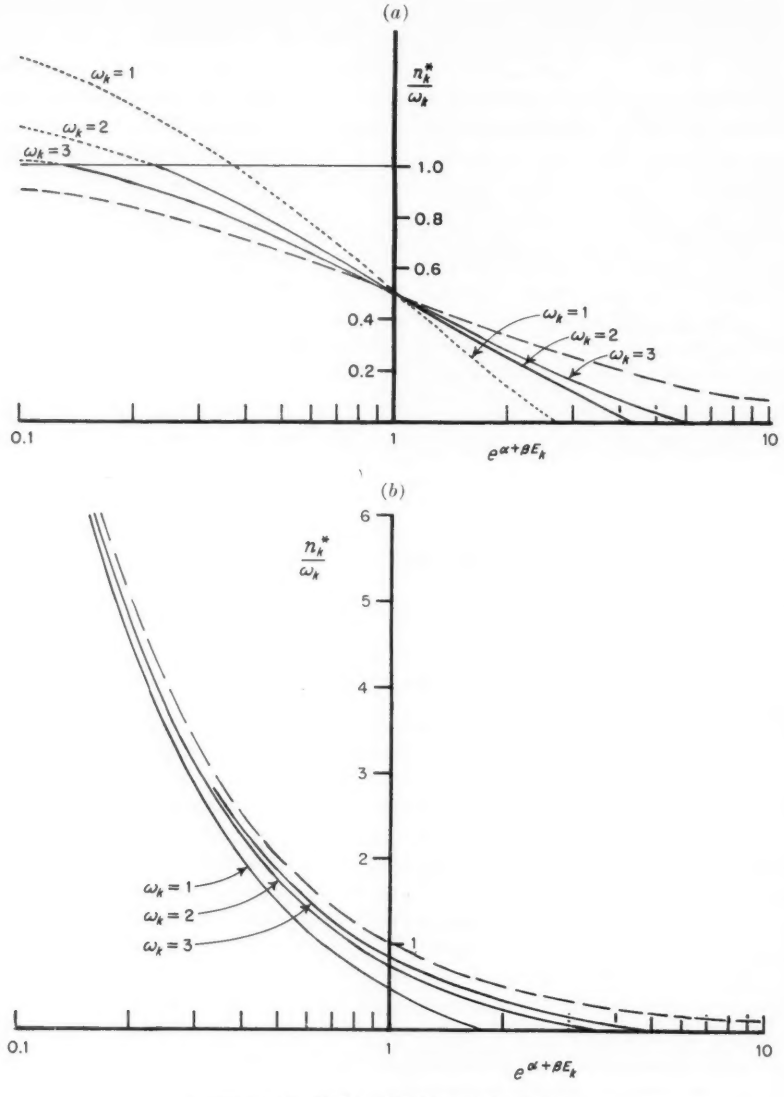


FIGURE 1 (*a* and *b*).—Caption on facing page.

$$G_k = \frac{\omega_k!}{n_k!(\omega_k - n_k)!}, \qquad \frac{\omega_k^{n_k}}{n_k!}, \qquad \frac{(n_k + \omega_k - 1)!}{n_k!(\omega_k - 1)!}. \qquad \text{(1a, b, c)}$$

Equation (1a) holds if only one particle is allowed in each of the $\omega_k$ states, and is, apart from constant factors, *the same whether the particles are distinguishable or not.* Equations (1b) and (1c) hold if the occupation of a state is unrestricted, the former if the particles are distinguishable, the latter if they are indistinguishable. We wish to determine the state ($n_1^*$, $n_2^*$, ...) of the whole system which can be realized in the largest number of different ways, subject to the restriction that $\Sigma_k n_k$ and $\Sigma_k n_k E_k$ are both fixed at $N$ and $E$, respectively. This state is usually referred to as the most probable state (the assumption of equal *a priori* probabilities of the various states of the whole system is then involved in the argument). We shall, as usual, make the assumption of continuity, and replace factorials by Gamma functions. One can then use undetermined multipliers $-\alpha$, $-\beta$, and finds that $n_k^*$ must be a solution of

$$\frac{\partial \log G_k}{\partial n_k} = \alpha + \beta E_k. \qquad (2)$$

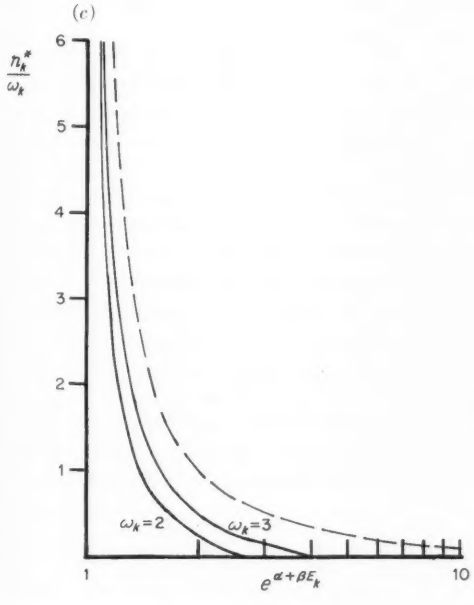


FIGURE 1

—— Most probable occupation numbers using the assumption of continuity
---- Mean occupation numbers on the basis of the grand canonical ensemble, *or* most probable occupation numbers using Stirling's approximation (5).

(*a*) For systems of Fermi-Dirac particles; (*b*) for systems of Maxwell-Boltzmann particles; (*c*) for systems of Bose-Einstein particles.

The resulting equations can be expressed in terms of Gauss's $\psi$-function,

$$\psi(n) \equiv \frac{d}{dn}\{\log[\Gamma(1+n)]\}, \tag{3}$$

no appeal being made to Stirling's approximation. By equations (1), (2), and (3),

$$\alpha + \beta E_k = \begin{cases} \psi(\omega_k - n_k^*) - \psi(n_k^*), & \text{(4a)} \\ \log \omega_k - \psi(n_k^*), & \text{(4b)} \\ \psi(n_k^* + \omega_k - 1) - \psi(n_k^*), & \text{(4c)} \end{cases}$$

corresponding to the three equations shown in equation (1). The results are plotted in figures 1 (*a*) to 1 (*c*) together with the curves which one obtains with the approximation

$$\psi(n) \sim \log n, \tag{5}$$

which corresponds to Stirling's approximation. $\alpha$ and $\beta$ may be identified in the usual way.

Several comments are required. (*a*) $\alpha + \beta E_k$ has been treated as a continuous variable, $n_k^*$ being the corresponding dependent variable. For a given value of $\alpha + \beta E_k$ the value of $n_k^*$ given by the graphs must therefore be approximated by the nearest integer. (*b*) For $\omega_k = 2$ and 3 parts of the curves in figure 1 (*a*) are shown dotted. These dotted sections correspond to solutions of (2) which are inadmissible since they imply $n_k^* > \omega_k$. The reason for this result may be seen by noting that if the curve of $\log G_k(n_k)$ against $n_k$ has its slope always in excess of a certain finite quantity $m_k$ (say) for $n_k \geqslant 0$, then (2) can have no admissible solutions for energies $E_k$ which satisfy $\beta E_k < m_k - \alpha$. The dotted parts of the curves apply to energy levels which satisfy this inequality. (*c*) If $\omega_k = 1$ then $G_k = 1$ for all admissible values of $n_k$ in (1a) and (1c). It follows that all states of the whole system are equally likely in these cases, and one cannot expect (2) to have admissible solutions. In fact, (4c) has no general solution if $\omega_k = 1$. Equation (4a) has, however, the solutions indicated by the dotted curve in figure 1 (*a*) and these must therefore be spurious. There are in this case two admissible values of $n_k$ (0 and 1), and the solutions $n_k^*$ of (2) depend on how the points $G_k(0) = 1$ and $G_k(1) = 1$ are joined. If they are joined by the straight line $G_k(n_k) = 1$, then there is in general no solution of (2). All the values shown in figure 1 (*a*) for $\omega_k = 1$ are thus due to the fact that these two points have been joined by a more complicated curve which involves Gamma functions. (*d*) These considerations make it desirable to check the validity of the curve based on (4a) for which $\omega_k = 2$. In this case $G_k = 1$ if $n_k = 0$ or 2, and $G_k = 2$ if $n_k = 1$, so that a possible continuous function is $G_k = 1 + \sin[\frac{1}{2}(\pi n_k)]$. This function leads in fact to a curve which is very similar to that shown in figure 1 (*a*). Nonetheless spurious results are possible, and are obviously due to the assumption of continuity, as will be shown in greater detail in section 3.

The curves based on (5) happen to coincide with the exact mean occupation numbers of the states as obtained by considerations based on the grand canonical ensemble, and, in general, very nearly with the mean occupation numbers based on the canonical ensemble. Figures 1 (*b*) and 1 (*c*) suggest therefore that, for all

values of $\omega_k$, the *most probable* occupation of a state is in these cases less than the *mean* occupation as averaged over an ensemble. It is easy to see that this must be universally true if the most common occupation number of a certain state in an ensemble is zero, since the ensemble average of the occupation number, must be affected by those systems of the ensemble for which the state of interest is occupied, and it must therefore be positive. On the other hand, if in figure 1 (*a*) the most common occupation number of a certain state in an ensemble has reached the maximum admissible one (i.e., unity), the mean occupation number, as averaged over an ensemble, must fall below this value, because of all those systems in the ensemble in which the state of interest is unoccupied.

For an approximate discussion of (4) note that

$$\psi(n) \sim \log n + \frac{1}{2n} - \frac{1}{12n^2} + \frac{1}{120n^4} - \ldots,$$

$$\log (n + {}^1/_2) \sim \log n + \frac{1}{2n} - \frac{1}{8n^2} + \frac{1}{24n^3} - \ldots,$$

so that $\psi(n) \sim \log (n + {}^1/_2)$ is an approximation whose error is less than 4% for $n \geqslant 1$, and can with advantage be used instead of (5), when it is found that

$$n_k^* \sim \begin{cases} \dfrac{\omega_k}{\lambda_k + 1} - \dfrac{1}{2}\dfrac{\lambda_k - 1}{\lambda_k + 1}, & \text{(6a)} \\ \dfrac{\omega_k}{\lambda_k} - \dfrac{1}{2}, & (n_k^* \geqslant 1) \quad \text{(6b)} \\ \dfrac{\omega_k}{\lambda_k - 1} - \dfrac{1}{2}\dfrac{\lambda_k + 1}{\lambda_k - 1}, & \text{(6c)} \end{cases}$$

where $\lambda_k \equiv \exp(\alpha + \beta E_k)$. These expressions are less accurate than (4), but the second terms make them more accurate than those usually quoted.

*3. The Elimination of the Assumption of Continuity (Special Cases).*—Assume for the present purpose that the number of energy levels exceeds the number of particles present, and that every level has the same degeneracy $\omega > 1$. The case $\omega = 1$ has already been discussed in section 2. If $N$ is fixed, but $E$ is not fixed, $G$ can be given its maximum possible value by allowing just one particle into each of any set of $N$ energy levels, whatever the value of $\omega$ and whichever of the three statistics is applicable. For one can always increase the value of $G$ by distributing the $t(>1)$ particles in an energy level so that levels, which would otherwise be unoccupied, are each occupied by a single particle. This result is due to the inequalities

$$\omega^t > \begin{cases} \omega!/t!(\omega - t)!, & \text{(7a)} \\ \omega^k/k!, & \text{(7b)} \\ (\omega + t - 1)!/t!(\omega - 1)!, & \text{(7c)} \end{cases}$$

where $\omega$ and $t$ are both integers which are greater than unity.

Let us now suppose that such a distribution has established itself in a system of equally spaced energy levels, $E_j = j\epsilon$, $j = 1, 2, \ldots$, and let $a$ and $r$ be integers

such that $a \geqslant 1$, $1 \leqslant r \leqslant N$. Suppose further that the $N$ singly occupied levels extend from $E_a$ to $E_{a+N}$, except for an unoccupied level $E_{a+r}$, all other levels being also unoccupied. The total energy of the system can easily be shown to be

$$E = [{}^1/_2N(N+1) + s]\epsilon, \text{ where } s \equiv aN - r. \tag{8}$$

$s$ can be arranged to be zero or an arbitrary positive integer by a suitable choice of $a$ and $r$. It follows that the distributions described have the greatest possible probability in all three statistics, provided $E$ is fixed at, or in excess of, ${}^1/_2N(N+1)\epsilon$. In general (i.e., unless $s$ is 0 or 1) one can generate equally probable distributions from any given distribution of this type by shifting a particle down into an empty level, and shifting another particle up into an empty level, so as to keep the total energy constant.

The most probable distributions discussed in this section escape notice as soon as the assumption of continuity is made. They are therefore of no importance if ensembles are used to calculate most probable, or mean, occupation numbers. The numbers involved are then so large that the assumption of continuity is in general justifiable. The present discussion shows therefore the extent to which discussions can be in error which do not use ensembles, but simply seek to determine most probable distributions for the system of interest. It also shows how to construct examples of systems, such that a set of the most probable occupation numbers of the particles in the system of interest (expressed more precisely: a set of occupation numbers such that no more probable set exists) is quite different from a set of mean occupation numbers, as averaged over an ensemble of duplicates of this system of interest.

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* On leave of absence from Department of Natural Philosophy, The University, Aberdeen, Scotland.

[1] Schrödinger, E., *Statistical Thermodynamics*, Cambridge University Press, 1948.
[2] Gurney, R. W., *Introduction to Statistical Mechanics*, McGraw-Hill, 1949.
[3] Tolman, R. C., *Statistical Mechanics*, Oxford University Press, 1938.
[4] Becker, R., *Z. Phys. Chem.*, **196**, 181 (1950).

# *RECENT TECHNICAL MANIFESTATIONS OF VON KÁRMÁN'S VORTEX WAKE*

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* *Read before the Academy, November 11, 1953*

When a fluid or gas flows past a cylindrical obstacle, the wake behind it is seen to contain numerous whirls or vortices arranged in a regular pattern, which was described and drawn accurately as early as the fifteenth century by Leonardo da Vinci. The whirls appear alternately on the one and on the other side of the cylinder, and they are washed away in the wake when they have reached a certain size. At the moment that a vortex has reached its maximum size, just before detaching itself from the cylinder, the velocity of the flow past that side of the cylinder is maximum and hence, by Bernoulli's law, the pressure on that side is a minimum. The cylinder thus experiences an alternating force in a direction perpendicular to that of the flow.

In 1878 Strouhal published a formula for the frequency of this force or of this vortex shedding, based on observations only. This formula is now usually represented in a dimensionless form: $S = fD/V$, where $S$ is the dimensionless "Strouhal number," a constant of value 0.22 or about 1/4.5; $f$ is the frequency, $D$ the cylinder diameter, and $V$ the stream velocity; the latter three quantities being expressed in any set of consistent units. This relation can be remembered by observing that one period of force oscillation (or the shedding of one pair of vortices) occurs during the time that it takes the stream to advance through 4.5 diameters.

In 1911 von Kármán made a stability analysis of the vortices and from it derived the geometrical pattern. This was the first theoretical investigation of the subject, and it is so important that the vortex wake now is generally referred to as the Kármán wake. The intensity of the alternating force can be written, for dimensional reasons, as:

$$F_K = C_K \rho \frac{V^2}{2} A$$

where $F_K$ is the Kármán force; $\rho V^2/2$ the stagnation pressure, $A$ the projected area of the obstacle (perpendicular to the stream), and $C_K$ a dimensionless coefficient, which may be called the Kármán coefficient. It is expected that this coefficient is a function of the Reynolds Number. Calculations have been made by various authors, none very satisfactory, indicating that $C_K \approx 1$, in other words, that the maximum value of the Kármán force is about the same as the stagnation force. Also, it is stated in the literature that for Reynolds Numbers over $3 \times 10^5$ for cylinders, the Strouhal formula no longer holds, and the vortex wake is much smaller, so that $C_K \ll 1$. The latter statement has been proved incorrect by recent observations of large industrial smokestacks vibrating violently at the correct Strouhal frequency with Reynolds Numbers as high as $7 \times 10^6$.

The above value of $C_K$ is based on the assumption that the cylinder or other obstacle does not vibrate. If it does vibrate, two effects have been noticed: First if the frequency of the cylinder vibration is in the neighborhood of the natural

Strouhal frequency (between 80 and 120 per cent of it) then the cylinder motion forces the vortex shedding to take place at the cylinder frequency, and not at the Strouhal frequency, and second, the intensity of the force is greater for a cylinder vibrating at the Strouhal frequency than for a cylinder at rest.

These two effects have been observed qualitatively only; no reliable numerical experimental results are available. The technical importance of airplanes is so very much greater than that of vibrating cylinders that not much attention has been paid to the intensity of the Kármán force so far. It is hoped that a comprehensive experimental investigation of the Kármán coefficient $C_K$ as a function of (1) Reynolds Number, (2) amplitude ratio $x$/diameter, and (3) frequency ratio $\omega_{cyl}/\omega_{Strouhal}$, will be carried out in the near future.

Technical applications of the Kármán wake have been telephone lines, electric cross-country transmission lines, submarine periscopes, television broadcasting antennas, industrial smokestacks, singing ship propellers, and the failure of one large suspension bridge. In all cases trouble is experienced when the natural frequency of the structure coincides more or less with the Strouhal frequency. A convenient measure for the relative intensity of the excitation in these various cases is the ratio between the exciting Kármán force and the dead weight of the vibrating object:

$$\frac{F_K}{W} = \frac{C_K(\rho_f V^2/_2)Dl}{\rho_b(\pi/4)D^2 l} = \frac{2}{\pi} C_K \left(\frac{\rho_f}{\rho_b}\right)\left(\frac{V^2}{gD}\right) = \text{const.} \left(\frac{\rho_f}{\rho_b}\right). \text{Frou.}$$

Here $\rho_f/\rho_b$ is the density ratio of the fluid and the vibrating body and the combination $V^2/gD$ is the dimensionless Froude number. In general, a light, stiff structure of large surface area is most susceptible to these vibrations. Of the various cases enumerated above, the first five have been described in the literature repeatedly, and the last two are dealt with in the papers referred to in footnotes 1 and 2. Here we discuss two recent cases of smokestack vibration. The first of these occurred in Monterrey, Calif., involving three stacks of 12-ft. diameter and 200-ft. height, made of welded steel. These stacks vibrated for several hours at the Strouhal frequency of about one cycle per second with large amplitudes at a Reynolds Number of about $2.0 \times 10^6$. No serious damage occurred, but to prevent recurrence, the stacks were provided with vibration dampers in the form of latticed struts, placed between the three stacks and from them to the adjacent building. One end of each strut was securely fastened, while at the other end a slipping clutch was provided. The slip force of this clutch was calculated to provide such damping of the motion that with an assumed $C_K = 1$ the resulting amplitudes would be small. Since the installation of these slip dampers two years ago, the vibration has not been observed.

The next case on record occurred in St. Clair near Detroit on several welded steel stacks of 300-ft. height and 16-ft. diameter at a Strouhal frequency of about a cycle per second and a wind velocity of about 45 miles per hour; the Reynolds Number being about $7 \times 10^6$. This resulted in a buckling of the structures in their upper part, so that they had to be taken down to about half height and built up again. They are being provided with dampers in three guy cables located at 120 deg. apart around the periphery near the top of the stack. Where these cables come near the ground large flexible springs are put in them, which are capable

of changes in length of some two feet. These springs and the cable are mounted in permanent tension, and a large truck-type automotive shock absorber is installed across the springs. Again the damping of these absorbers is chosen so that their energy dissipation is somewhat greater than the expected energy input from the vortices, assuming a coefficient $C_K = 1$.

In conclusion it can be stated that welded steel stacks of the size described or larger stacks are unsafe, and that dampers or other means of suppressing the vibrations are essential.

[1] Kerr, Shannon, and Arnold, "The Problems of the Singing Propeller," *Inst. Mech. Engrs. Proc.*, **144**, 54 (1940).

[2] Farquharson, "Aerodynamic Stability of Suspension Bridges, the Tacoma Narrows Bridge," *Univ. Wash. Eng. Exp. Sta. Bull.*, No. 116, Part III.

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## *HIGH-STRENGTH PLASTICS*

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*General.*—The term "high-strength plastics" refers to composite materials in which plastics act as binders, and high-strength fibers provide most of the strength properties. As a consequence, the composite has mechanical properties unavailable in either constituent.

Strength, toughness, rigidity, resilience, and flexibility are controlled by the selection and proportioning of fibers and resins; consequently, these materials range from soft and flexible but tough and strong, to hard, rigid, and relatively inextensible. The judicious combination of resins and fibers can provide high strength-to-weight ratios; the strong fibers can be oriented in the directions of maximum stresses; structural forms can be tailored to fit the requirements of a given application; and special requirements such as transparence to electromagnetic radiation, resistance to various corrosive conditions, high energy absorption, and abrasion resistance can be met.

A few of the diverse applications are:

1. Molded seamless boat hulls of glass fiber mat combined with polyester resins.
2. Lightweight bodies for automobiles. Good strength and resilience are combined with relatively low tooling costs for limited production such as sports cars.
3. Radar housings, especially in the form of structural sandwiches for strength, rigidity, and minimum interference with radar propagation.
4. Body armor. In Korea, this spectacularly successful application has prevented a large number of casualties, the great majority of which are caused by flying fragments from shell bursts. Heavy nylon fabric bonded with just sufficient phenolic resins to hold the plies together affords good resistance to penetration by such missiles. Laminated glass fabric is also highly effective for this application. Here high strength and high energy absorption are both important.

Other applications include lightweight strong pipe, nonmetallic disk springs, fishing rods, corrugated sheet for buildings, truck tanks, and many other similar applications involving lightness, strength, resilience, and toughness.

*Engineering Design.*—Engineering design depends upon a knowledge of the properties of both reinforcement and resin, particularly their stress-strain relationships and their behavior under long-continued load or deformation. Many of these properties still require extensive study.

High-strength plastics fall into two general categories. High-pressure laminates employ such resins as phenol-formaldehyde and melamine formaldehyde, and require high heat and pressure during fabrication. Low-pressure laminates, now called reinforced plastics, employ polyesters or epoxies, require little or no forming pressure, and temperatures are moderate during fabrication.

A variety of reinforcements may be employed but the most common are glass fiber and nylon. Glass fiber has extremely high strength but is relatively inextensible whereas nylon has good strength combined with high extensibility.[1] The reinforcing material may be employed in a variety of forms: parallel fibers in the form of roving; random mats of short-length fibers; and woven fabrics including square weave, four- and eight-shaft satin weave, and unidirectional fabrics are most common.

The tensile strengths of the resins are much lower than those of the reinforcing agent, nevertheless the resins perform the essential function of binding the fibrous materials together, providing them with the necessary lateral stability under compressive loads and developing the shearing rigidity of the combination necessary for engineering applications.

Strength properties of a given combination of resin and fiber depend upon the relative proportions of each; the structure of the reinforcement, whether mat, woven fabric, or straight filament; and the manner in which they are combined. Tensile strengths, for example, may vary from approximately 10,000 psi for ordinary glass mat reinforced materials to as high as 200,000 to 300,000 psi for rods incorporating a high proportion of carefully aligned fine glass filaments.

In making engineering computations, it is generally assumed that the stress is divided between resin and reinforcement in proportion to the ratio of their moduli of elasticity. This assumption, in turn, depends not only upon the stress level but upon the relaxation and creep behavior of both resin and reinforcement under continuous deformation or continuous load. Little is known about these characteristics and the design assumptions are consequently uncertain to that extent.

Because of the directional characteristics of the reinforcing material, these high-strength plastics are generally not isotropic, and the simple theory of isotropic elastic behavior consequently does not apply. Fortunately, the cumbersome general theory of non-isotropic materials can usually be simplified to the theory of two-dimensional orthotropic materials, that is, materials with two principal axes oriented at right angles. This theory involves five constants: two moduli of elasticity parallel to the two principal directions, the sharing modulus, and two values of Poisson's ratio associated with the two principal directions. Given these constants, the values of modulus of elasticity, shearing modulus, and Poisson's ratio can be computed for intermediate directions.

The relationships for design of orthotropic material[2, 3] are compared with isotropic materials in figure 1, in which tensile and shearing stresses are shown applied to a particle. This particle is shown with two principal axes $L$ and $T$, with direct stress $\sigma_1$ applied in the 1-direction at an angle $\alpha$ with the $L$-axis. Shear stress $\tau_{12}$

is associated with the 1-2 plane. In an isotropic particle there are no principal axes because properties are the same in all directions. For isotropic materials the three simple relationships are shown on the left. The modulus of elasticity $E$, the shearing modulus $G$, and Poisson's ratio $\mu$ do not vary with angle of stress. The expressions for strain in terms of stress, elastic moduli, and Poisson's ratio are simple. For orthotropic materials, however, as the direction of stress varies from parallel to one

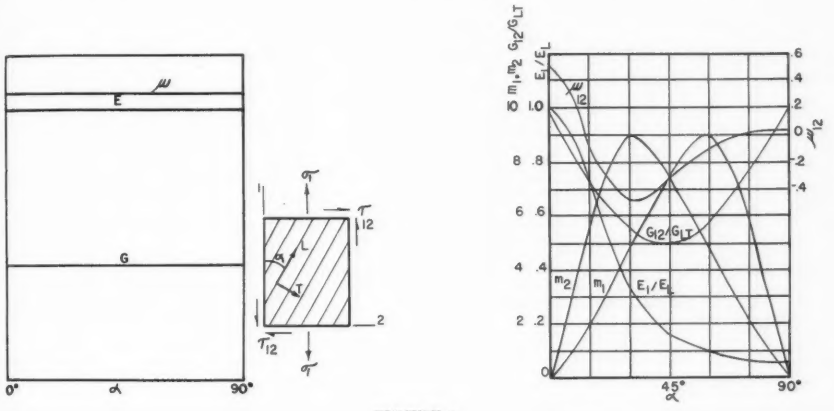


FIGURE 1

Values of elastic constants as functions of orientation. In isotropic materials, left, modulus of elasticity, shearing modulus, and Poisson's ratio are independent of orientation. In orthotropic materials, right, these values change with orientation, and strains associated with a given stress are strongly dependent upon its orientation.

| ISOTROPIC MATERIALS (*Left*) | ORTHOTROPIC MATERIALS (*Right*) |
|---|---|
| Strains caused by $\sigma_1$ | |
| $e_1 = \sigma_1/E$ | $e_1 = \sigma_1/E_1$ |
| | $E_L/E_1 = \cos^4\alpha + E_L/E_T \sin^4\alpha + {}^1/_4(E_L/G_{LT} - 2\,\mu_{LT}) \sin^2 2\alpha$ |
| $e_2 = -\mu e_1$ | $e_2 = -\mu_{12} e_1$ |
| | $\mu_{12} = E_1/E_L\{\mu_{LT} - {}^1/_4(1 + 2\,\mu_{LT} + E_L/E_T - E_L/G_{LT}) \sin^2 2\alpha\}$ |
| | $\gamma_{12} = -m_1\sigma_1/E_L$ |
| | $m_1 = \sin 2\alpha\{\mu_{LT} + E_L/E_T - {}^1/_2\, E_L/G_{LT} - \cos^2\alpha(1 + 2\,\mu_{LT} + E_L/E_T - E_L/G_{LT})\}$ |
| Strains caused by $\tau_{12}$ | |
| $\gamma_{12} = \tau_{12}/G$ | $\gamma_{12} = \tau_{12}/G_{12}$ |
| $G = E/2(1 + \mu)$ | $G_{LT}/G_{12} = G_{LT}/E_L\{(1 + 2\,\mu_{LT} + E_L/E_T) - (1 + 2\,\mu_{LT} + E_L/E_T - E_L/G_{LT}) \cos^2 2\alpha\}$ |
| | $e_1 = -m_1\tau_{12}/E_L$ |
| | $e_2 = -m_2\tau_{12}/E_L$ |
| | $m_2 = \sin 2\alpha\{\mu_{LT} + E_L/E_T - {}^1/_2\, E_L/G_{LT} - \sin^2\alpha (1 + 2\,\mu_{LT} + E_L/E_T - E_L/G_{LT})\}$ |

principal direction to parallel to the other, the modulus of elasticity $E_1$ changes from $E_L$ at 0° to $E_T$ at 90°, the shearing modulus $G_{12}$ is equal to $G_{LT}$ at 0° and 90° but drops to a minimum at 45°, and the Poisson's ratio $\mu_{12}$ changes markedly from $\mu_{LT}$ at 0° to $\mu_{TL}$ at 90°, even becoming negative at some orientations. Furthermore, at any orientation except the principal directions, the direct stresses cause shear distortion and the shear stresses cause changes in length and width proportional to the values $m_1$ and $m_2$ shown.

The foregoing relationships apply to single orthotropic layers. When these are laminated at various orientations to each other, stresses applied to the entire assemblage cause interactions between the layers which may develop high direct stresses in some layers and low stresses in others, and may develop shear stresses in and between layers even when no external shear stresses occur.

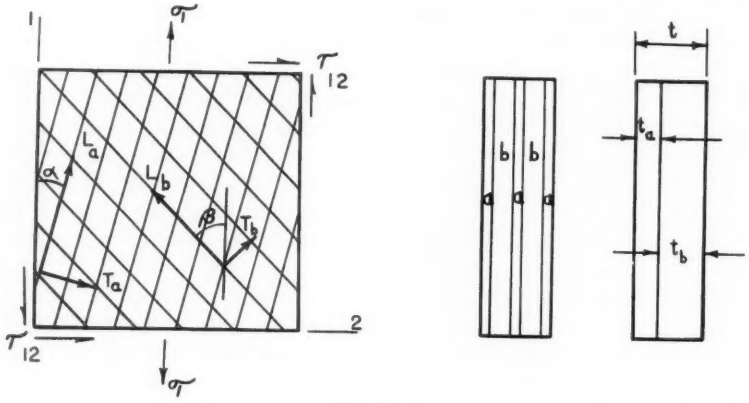


FIGURE 2

Laminated particle with layers $a$ and $b$ oriented at arbitrary angles $\alpha$ and $\beta$ with direction 1.

In lamina $a$

For direct stress $\sigma_1$ alone

$$e_{1a} = \sigma_{1a}/E_{1a} - \mu_{21a}\sigma_{2a}/E_{2a} - m_{1a}\tau_{12a}/E_{La} \quad (1a)$$

$$e_{2a} = -\mu_{12a}\sigma_{1a}/E_{1a} + \sigma_{2a}/E_{2a} - m_{2a}\tau_{12a}/E_{La} \quad (1b)$$

$$\gamma_{12a} = -m_{1a}\sigma_{1a}/E_{La} - m_{2a}\sigma_{2a}/E_{La} + \tau_{12a}/G_{12a} \quad (1c)$$

$$\sigma_{1a}t_a + \sigma_{1b}t_b = \sigma t \quad (2a)$$

$$\sigma_{2a}t_a + \sigma_{2b}t_b = 0 \quad (2b)$$

$$\tau_{12a}t_a + \tau_{12b}t_b = 0 \quad (2c)$$

$$e_{1a} = e_{1b} = e_1 \quad (3a)$$

$$e_{2a} = e_{2b} = e_2 \quad (3b)$$

$$\gamma_{12a} = \gamma_{12b} = \gamma_{12} \quad (3c)$$

$$A_{11}\sigma_{1a} + A_{12}\sigma_{2a} + A_{13}\tau_{12a} = (\sigma/E_{1b})(t/t_at_b) \quad (4a)$$

$$A_{21}\sigma_{1a} + A_{22}\sigma_{2a} + A_{23}\tau_{12a} = -(\mu_{12b}\sigma/E_{1b})(t/t_at_b) \quad (4b)$$

$$A_{31}\sigma_{1a} + A_{32}\sigma_{2a} + A_{33}\tau_{12a} = -(m_{1b}\sigma/E_{Lb})(t/t_at_b) \quad (4c)$$

in which

$$A_{11} = \frac{1}{E_{1a}t_a} + \frac{1}{E_{1b}t_b} \quad A_{22} = \frac{1}{E_{2a}t_a} + \frac{1}{E_{2b}t_b} \quad A_{33} = \frac{1}{G_{12a}t_a} + \frac{1}{G_{12b}t_b}$$

$$A_{12} = A_{21} = -\frac{\mu_{12a}}{E_{1a}t_a} - \frac{\mu_{12b}}{E_{1b}t_b}$$

$$A_{13} = A_{31} = -\frac{m_{1a}}{E_{La}t_a} - \frac{m_{1b}}{E_{Lb}t_b}$$

$$A_{23} = A_{32} = -\frac{m_{2a}}{E_{La}t_a} - \frac{m_{2b}}{E_{Lb}t_b}$$

The theoretical relationships between the interactions of several orthotropic layers $a$ and $b$ bonded together at an arbitrary angle $\alpha + \beta$, are shown in figure 2. Layers $a$, of total thickness $t_a$, are oriented at angle $\alpha$ with the 1-direction, and layers $b$, of total thickness $t_b$, are oriented at the reverse angle $\beta$ with the 1-direction. For each lamina a separate solution of stresses in the 1-2 directions is possible.

Equations (1) through (4) give the relationships from which stresses $\sigma_{1a}$, $\sigma_{2a}$, and $\tau_{12a}$ in the $a$ layer, caused by stress $\sigma_1$, can be computed. Equations (1) set up the relationships between stress and strain in the $a$ layer, equations (2) state that the

summation of internal stresses in the layers $a$ and $b$ must be equal to the external stresses, and equations (3) state that the strains in all layers are equal, and equal to strains in the entire particle. From these equations are derived the simultaneous equations (4) which lead to solutions for

$$\sigma_{1a}, \sigma_{2a}, \text{ and } \tau_{12a}.$$

From the foregoing it may be seen that the engineering design of materials of this type is feasible but is more complex than the design of isotropic materials. In practice many problems still remain to be solved. There have been some spectacular successes with these materials. There have also been some mystifying failures. These can probably be ascribed to a number of different factors of which present knowledge is far from complete. They are:

1. The meagerness of knowledge of the elastic constants. Generally, tensile strength properties including modulus of elasticity are known in some principal direction. Little is known about shearing modulus and practically nothing about Poisson's ratio. A great deal of experimental work is needed to develop these constants. To this must be added the effects of creep and relaxation.

2. Many engineers are unfamiliar with the theory of orthotropic materials and, consequently, attempt to design on the basis of the theory of elasticity for isotropic materials. This may easily lead to neglect of important stresses.

3. The tacit assumption is made that there is perfect bond between the binder and the reinforcement. It is well known that this is not necessarily true. One of the major problems facing this field is the problem of adhesion between resin and fiber. Extensive and active research is under way to develop treatments for the fibers, particularly glass, to insure good adhesion to the resin. Active research is also under way to find resins which inherently bond well to the fiber. Bond is particularly important in pressure applications such as pipe in which some failures have occurred, probably because the fluid under pressure penetrated into the pipe wall along the fibers.

4. Fabricating techniques need to be improved. Slow and cumbersome hand lay-ups are often variable in quality. Because many of these materials, particularly those reinforced with glass fiber, are only slightly extensible, it is impossible to employ draw-forming operations such as those employed with metals. Considerable effort is being expended to see whether pre-impregnated sheets can be placed between forming dies and readily shaped. New and imaginative approaches to rapid and simple fabrication are needed.

5. High cost of the base materials, in turn, leads to high cost of finished items. Neither the fibers nor the resins can be considered cheap, particularly in relationship to carbon steel. Consequently, every pound of the material must be designed to work to its utmost to justify the cost.

In spite of these limitations, the high-strength plastics have certain inherent advantages which can be utilized in engineering design. They are light in weight for their strength, they can be fabricated into a wide variety of forms, and they allow the engineer to orient his high-strength elements to coincide with the stresses and thereby permit him to tailor his structures in terms of the engineering requirements. They are a challenge to the imagination and ingenuity of research men and of engineers.

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[1] Backer, S., and Susich, G., "Tensile Recovery Behavior of Textile Fibers," R&D Report, Textile Series No. 64, Department of the Army, Office of the Quartermaster General, November 1950.

[2] Hoff, N. J., Chapter 1 of *Engineering Laminates*, edited by A. G. H. Dietz, J. Wiley & Sons, Inc., New York, 1949.

[3] Love, A. E. H., *Mathematical Theory of Elasticity*, 4th ed., Dover Co., New York, 1927.

# *THE EFFECTS OF DNA AND ENZYME-TREATED DNA ON BACTERIAL POPULATION CHANGES*

By Werner Braun and Jeanne Whallon

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*Communicated by T. M. Sonneborn, January 16, 1954*

Desoxyribose nucleic acid (DNA) is suspected of a fundamental role in controlling specific biological activities, which includes its implied genetic function as a component of the chromosomal system and its ability to cause specific transformations of the genotype in various bacterial species.[1] In view of such implications, any demonstration of less specific effects of DNA upon changes in the genotypic composition of a cell population becomes of interest. Such less specific effects of DNA were observed in recent studies with Brucella populations which attempted to determine the possible existence of phenomena akin to transformation among Brucella strains.

DNA was isolated from Brucella cells by two procedures: (*a*) by extracting cells, treated with desoxycholate, according to the procedure of McCarty and Avery,[2] and (*b*) by a similar procedure (deproteinization and alcohol precipitation) applied to the supernatant of heavy cell suspensions in 0.5% phenol, which had been shaken for 48 hours at 37°C. in the presence of glass beads. The activity of the two preparations was equal, but the yields of highly purified DNA, identified as such by physical and chemical methods (ultra-violet absorption, solubility, Dische-Stumpf reaction, hydrolysis and chromatography), were far greater by procedure (*b*). Population changes in liquid media, in the presence or absence of DNA preparations, were determined by spreading a sample of the culture on agar where the phenotypes (smooth or *S*, rough or *R*, mucoid or *M* morphology) of the resulting colonies indicated the frequency in the population of cells with altered genotype.[3] Early results with a number of *B. suis*, *B. abortus*, and *B. melitensis* strains indicated that initially homogeneous broth cultures supplemented with heterologous DNA tended to show slightly enhanced population changes involving the establishment of a variety of colonial types, including mutants resembling the type from which the DNA had been obtained. In contrast, addition of homologous DNA tended to suppress population changes. In order to test the dependence of these effects upon DNA, desoxyribonuclease (DNase) was added to cultures that had been supplemented with either heterologous or homologous DNA. Under such conditions certain strains exhibited very extensive population changes, whereas control cultures without DNA + DNase, without DNA, or without DNase, failed to exhibit changes of comparable extent (for example, see table 1). Since these striking population changes occurred even after addition of DNase to cultures containing homologous DNA, it was suspected that the effects of enzyme-treated DNA and the above-cited effects of heterologous DNA alone, might involve different mechanisms. The effects produced by enzyme-treated, presumably depolymerized DNA, therefore were investigated more thoroughly and the results obtained in such studies represent the major subject of this communication.

*B. abortus*, Strain 19 *M* (mucoid) cells were used as inoculum in all studies on the effects of DNA + DNase, and the establishment of *S* (smooth) cells was determined by streaking samples from aging beef-extract broth cultures on 2-1 agar.[3] In

most experiments supplementation with 150 $\gamma$ DNA and 75 $\gamma$ DNase per ml. was employed. Table 1 presents a typical result, showing the high degree of population change occurring in cultures supplemented with DNA + DNase. Experiments in which the DNase was inactivated, prior to addition to the culture medium, by brief boiling of the DNA + DNase mixture after various intervals following the addition of DNase, revealed that a very brief exposure to DNase (less than 5 seconds) sufficed to produce the sufficiently altered state of DNA responsible for the effect. Control experiments showed that similar boiling of DNA alone produced no significant effects. Purines and pyrimidines (xanthine, hypoxanthine, guanine, adenine, uracil, thymine), as well as acid hydrolysates of bacterial DNA, were found entirely ineffective in duplicating the effects of presumably slightly depolymerized

TABLE 1

THE EFFECTS OF DNA, DNase, AND DNA + DNase UPON POPULATION CHANGES OF INITIALLY *M* CULTURES OF BRUCELLA ABORTUS GROWN IN BUFFERED BEEF-EXTRACT BROTH

| MEDIUM | EXTENT OF POPULATION CHANGES AFTER: 3 DAYS | 5 DAYS | 9 DAYS |
|---|---|---|---|
| Control | <0.001% *S* | <1% *S* | <1% *S* |
| DNase | <0.01% *S* | 2% *S* | 4% *S* |
| DNA | <0.01% *S* | 3% *S* | 6% *S* |
| DNA + DNase | <1% *S* | 33% *S* | 60% *S* |

TABLE 2

RESULTS OF A TEST ON THE EFFECTS OF DNA, DNase, AND DNA + DNase UPON MUTATION RATES FROM STREPTOMYCIN SUSCEPTIBILITY TO STREPTOMYCIN RESISTANCE

Approximately $5 \times 10^9$ cells treated for 6 hrs. at 37°C., and appropriate controls, were plated on plain agar, covered by a protective layer of soft (0.7%) agar; a layer of soft streptomycin-containing agar was poured on top either immediately or 12 hrs. later; following application of the streptomycin layer the plates were kept at 5°C. for 12–18 hrs., to permit diffusion of the antibiotic, and were subsequently incubated at 37°C. for 6 days before scoring of the growth of resistant mutants.

| TYPE OF TREATMENT | AVERAGE NUMBER[a] OF RESISTANT COLONIES THAT DEVELOPED WHEN STREPTOMYCIN WAS ADDED AT: 0 HR. | 12 HRS. |
|---|---|---|
| None | 0 | 4 |
| DNA | <1 | 2 |
| DNase | <1 | 6 |
| DNA + DNase | 5 | 5 |

[a] Average of triplicate tests.

DNA. The effect is not specific for Brucella DNA, isolated from various smooth and nonsmooth strains, but was also obtained in Brucella cultures to which *E. coli* DNA (obtained by procedure (*b*) above) + DNase had been added. Highly polymerized commercial DNA (Worthington), similarly exposed to DNase, however, showed little activity. A number of appropriate tests, involving the determination of mutation rates from $M \rightarrow S$ as well as from streptomycin susceptibility to streptomycin resistance, demonstrated that the enzyme-treated DNA, affecting population changes, does not possess any obvious mutagenic activity. However, these tests appear to confirm previous observations with *E. coli*[4] in regard to a shortening of phenotypic lag in the presence of nucleic acid (see table 2). This shortened phenotypic lag did not suffice to explain the observed effects of DNA + DNase

upon population changes. Additional mechanisms responsible for the non-specific effect of presumably partly depolymerized DNA upon enhancing population changes were elucidated by a more thorough examination of the growth of *S* and *M* cells by themselves, or in mixture, in the presence or absence of DNA + DNase. As figure 1 illustrates, it was found that the presence of DNA + DNase does not modify the growth of *M* or *S* cells when they are by themselves. However, a pronounced inhibition of the growth of *M* cells, even subsequent "killing" of such cells, occurs in the presence of DNA + DNase when *S* cells are present, thus endowing the latter cells with a high selective value in mixed *M* + *S*, DNA + DNase-containing cultures. Furthermore, it was observed that the initial addition of more than 1 per cent *S* cells to *M* cultures promotes the gradual but relatively slow es-

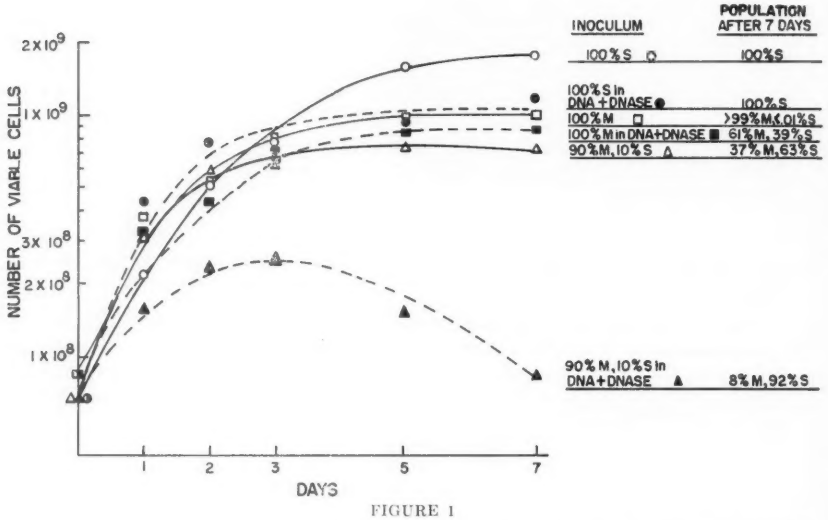
INOCULUM
POPULATION AFTER 7 DAYS
100% S ○ 100% S
100% S in DNA + DNASE ● 100% S
100% M □ >99% M, <01% S
100% M in DNA + DNASE ■ 61% M, 39% S
90% M, 10% S △ 37% M, 63% S
90% M, 10% S in DNA + DNASE ▲ 8% M, 92% S
NUMBER OF VIABLE CELLS
2 X 10^9
1 X 10^9
3 X 10^8
2 X 10^8
1 X 10^8
1
2
3
5
7
DAYS

FIGURE 1

Growth curves (viable cell counts) of initially *M*, *S*, or *M* + *S* cultures of *Brucella abortus* in the absence (——) and presence (– – –) of DNA + DNase. Wherever population changes occurred, i.e., in initially *M* and *M* + *S* cultures, the graph depicts the number of *M* cells only. (The count of *S* cells, growing in the presence of *M* cells, increased in the same way regardless of whether DNA + enzyme were added or not.)

tablishment of *S* cells even in the absence of DNA + DNase; in contrast, when such *S* cells are present initially in a proportion of less than 1 per cent they never establish themselves in aging cultures. Also, transfer of cultures at different intervals from media containing DNA + DNase to plain media will enhance the subsequent extent of *M*→*S* population changes in comparison to control cultures, but the rate of changes in such transferred cultures is less than in parallel cultures containing DNA + DNase. On the basis of these observations at least three factors may be held responsible for enhanced population changes in the presence of DNA + DNase: (1) shortening of the phenomic lag leading to the earlier establishment of phenotypically and genotypically altered cells; (2) a significant inhibitory effect upon the parent cells in the presence of a certain number of mutant cells

whenever enzyme-treated DNA is present; and (3) an additional ability of mutant cells to attain a positive selective value as soon as they are present in sufficient proportions, an effect which, as indicated by the "initial mixture" and "transfer" experiments, may be quite independent of the presence of the DNA "derivative." The nature of the inhibitory factor(s) produced by *S* cells in the presence of DNA + DNase is unknown, but may involve a metabolite since the effectiveness of the DNA + DNase effect depends not only upon the strain but also upon the type of medium employed. Finally, the ability of DNase alone to enhance population changes slightly (table 1) may indicate a normal accumulation of DNA in the culture medium which is subsequently converted into "active" DNA by the presence of DNase.

It remains to be determined whether the described non-specific effects of a DNA-like substance upon bacterial population changes might play a secondary role in the more specific phenomenon of bacterial transformations caused by highly polymerized DNA. A more comprehensive report of these studies will be published elsewhere.

[1] Austrian, R., *Bact. Rev.*, **16**, 31 (1952).
[2] McCarty, M., and Avery, O. T., *J. Exptl. Med.*, **83**, 105 (1946).
[3] Braun, W., *Am. Naturalist*, **86**, 355 (1952).
[4] Witkin, E., and Flint, J., in *Carnegie Institution Year Book*, **48**, 161 (1949).

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## *THE PRODUCTION OF MUTATIONS IN DROSOPHILA MELANOGASTER BY THE FAST NEUTRON RADIATION OF AN ATOMIC EXPLOSION**

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*Communicated by Karl Sax, January 26, 1954*

Recently the United States Atomic Energy Commission gave us the unusual opportunity of making a small scale study of the effects of the high intensity fast neutrons produced by an atomic explosion on the mutation rate in the mature sperm of *Drosophila melanogaster*. The males were shielded during the explosion so as to avoid temperature extremes, blast, and nearly all of the other radiation produced by the explosion. Dosimeters were included with the flies, and the estimated dosage received by the flies was calculated and given to us in terms of Roentgens Equivalent Physical (REP).

In the laboratory within a week after exposure the radiated males were mated individually in part to Muller-5 females and in part to *res* females, *res* being a combination of eight recessive third chromosome phenotypic mutations used in a similar test of x-rays by Alexander.[1] The offspring of the Muller-5 cross were used for sex-linked lethal tests by Levine. The offspring of the *res* cross were classed by Ives for four groups of visible mutations: *res*-like (resembling one or another of the eight mutants of that stock), Minute (thin bristles), other bilateral mutants, and unilateral mutants of all kinds. The recovered sex-linked lethals were tested by

Ives and Levine for crossing-over with *y ct*$^6$ *ras*$^2$*f*, a group of four sex-linked visibles well spread out in the *X*-chromosome. This showed how many of the lethal chromosomes gave normal crossing-over and how many gave abnormal crossing-over of a type generally associated with a chromosome inversion or translocation. It also proved that in the present instance all lethals derived from a given male were in each case independent mutations.

Altogether this made an unusually comprehensive test of the genetic effects of the radiation received. While the data are not large in comparison with many Drosophila experiments, it seems best to report them in their present form, particularly since it is very unlikely that both intensity and dosage, to say nothing of other conditions, can be even approximately duplicated in another atomic explosion in the near future.

The stock of normal flies used was a strain known as Oregon-R, which had been inbred by single pairs for 100 generations and then kept in mass cultures for about 10 generations before the exposure to atomic radiations. During those 10 generations a similar analysis had been made of soft x-rays by Ives and Yost, using the *res* stock only. In the previous year[2] an earlier derivative of the same inbred line had been tested for sex-linked mutation rate with soft x-rays. The data of the x-ray *res* tests and some of the results of the x-ray sex-linked lethal tests are included

TABLE 1

SEX-LINKED LETHAL MUTATION RATES

| DOSAGE | TESTS | LETHALS | PER CENT |
|---|---|---|---|
| 315 REP | 1453 | 45 | 3.1 |
| 760 REP | 1000 | 61 | 6.1 |
| 1400 REP | 425 | 48 | 11.3 |
| 2500 r x-rays | 2403 | 165 | 6.9 |

here for comparison with the effects of high-intensity fast neutrons. Conditions and criteria for the sex-linked lethal determinations in the present study were similar to those in the earlier study.[2] Radiation in the x-ray study of visibles using *res* was also from the same source as in the previous work (120 KV, 10 ma) at a rate of 200 r per minute.

Table 1 shows the sex-linked lethal mutation rates observed in this study. Included for comparison is the mutation rate of 2500 r soft x-rays found by Young and us and reported earlier.[2] The table shows that 760 REP of high-intensity fast neutrons produced by an atomic bomb are comparable in effect to 2500 r of low intensity soft x-rays. On the basis of this dosimetry the fast neutrons were $3^1/_2$ times as effective as the x-rays.

Table 2 shows the results of analyzing the lethals by means of *y ct*$^6$ *ras*$^2$*f*. Most of the lethals of the fast neutron series were successfully analyzed by this method. But only a small random sample of the soft x-ray lethals were similarly analyzed in the earlier study. Also included for comparison is a series of lethals appearing in a study with a naturally occurring mutator and published previously.[3] Any lethal chromosome showing either allelism to one of the marker genes in the $F_1$ of this test or abnormal crossing-over in both the males and females of the $F_2$ were classed as complex. The others were classed as simple. Crossing-over was con-

sidered as abnormal when in a count of not less than 100 flies it was reduced to less than half the map distance in one or more regions of the chromosome. Only a few cases of allelism were found and most of them were also associated with reduced crossing-over. No cytological study of radiation lethals has been attempted, but an earlier study[4] showed that the complex lethals in the mutator series were cases of gross chromosomal rearrangement, chiefly inversions.

The data of table 2 show that a large portion of the lethals in the fast neutron series were complex mutations. In practically all cases these showed marked reduction in crossing-over in one or more regions of the chromosome. The proportion did not change significantly from one dosage to another and averaged 42 per cent in the three dosages together. Data on the distribution of these chromosome aberrations in terms of the cross-over regions involved are not included in this report. It may be said, however, that in general the distribution appears to be random. A large portion of them involved the *ras-f* region, as should be the case, since the

TABLE 2

FREQUENCY OF COMPLEX LETHALS

| DOSAGE | LETHALS | SIMPLE | COMPLEX | COMPLEX, % |
|---|---|---|---|---|
| 315 REP | 39 | 21 | 18 | 46 |
| 760 REP | 57 | 35 | 22 | 39 |
| 1400 REP | 43 | 24 | 19 | 44 |
| TOTAL | 139 | 80 | 59 | 42.4 |
| 2500 r | 40 | 34 | 6 | 15.0 |
| Mutator | 351 | 332 | 19 | 5.4 |

TABLE 3

PROPORTIONS OF SIMPLE AND COMPLEX LETHALS

| DOSAGE | LETHALS, % | SIMPLE | COMPLEX |
|---|---|---|---|
| 315 REP | 3.1 | 1.7 | 1.4 |
| 760 REP | 6.1 | 3.7 | 2.4 |
| 1400 REP | 11.3 | 6.4 | 4.9 |
| 2500 r | 6.9 | 5.8 | 1.1 |

large heterochromatic region of the chromosome is entirely beyond *f*. In some cases, however, recovered cross-overs were of normal proportion only in the *ras-f* region, indicating that the neutron bombardment also caused breaks in the euchromatic region of the chromosome.

The fast neutron series had far more complex lethals than appeared in either of the other two series, three times as many as the x-ray series and eight times as many as the mutator series. The number of lethals tested in the x-ray series was not large enough to make the comparison a completely satisfactory one. In both comparisons, however, the chi-square *P* value (with Yates correction) is less than one per cent, indicating a statistically significant difference between the fast neutron series and each of the other two.

Using the lethal rates in table 1 and the percentage of complex lethals in table 2, it is possible to divide the total portion of lethals in each radiated series into two parts, simple lethals and complex lethals as in table 3. The comparison is again a rough one. It suggests that 1400 REP of fast neutrons was approximately equiva-

lent to 2500 r of x-rays in producing simple lethals, while 315 REP equaled 2500 r in producing complex lethals. In the one case, neutrons were twice as effective as x-rays; in the other they were eight times as effective.

TABLE 4

VISIBLE MUTATIONS HIGH-INTENSITY IN FAST NEUTRON SERIES

| | | DOSAGE IN REP: | | | |
|---|---|---|---|---|---|
| | | 315 | 760 | 1400 | TOTAL |
| | | FLIES: | | | |
| CLASS | TYPE | 2250 | 1445 | 103 | 3798 |
| 1 | *res*-like | | | | |
| | No. | 10 | 6 | 0 | 16 |
| | % | 0.44 | 0.42 | 0 | 0.42 |
| 2 | Minutes | | | | |
| | No. | 11 | 12 | 5 | 28 |
| | % | 0.49 | 0.83 | 4.9 | 0.73 |
| 3 | Other bilat. | | | | |
| | No. | 14 | 16 | 4 | 34 |
| | % | 0.62 | 1.11 | 3.9 | 0.89 |
| 4 | Unilat. | | | | |
| | No. | 15 | 12 | 4 | 31 |
| | % | 0.67 | 0.83 | 3.9 | 0.81 |
| | Total | | | | |
| | No. | 50 | 46 | 13 | 109 |
| | % | 2.2 | 3.2 | 12.6 | 2.85 |

TABLE 5

VISIBLE MUTATIONS IN SOFT X-RAY SERIES

| | | DOSAGE IN r: | | | | |
|---|---|---|---|---|---|---|
| | | 3000 | 5000 | 7500 | 10,000 | TOTAL |
| | | FLIES: | | | | |
| CLASS | TYPE | 13,301 | 5,130 | 672 | 393 | 19,496 |
| 1 | *res*-like | | | | | |
| | No. | 68 | 32 | 12 | 3 | 115 |
| | % | 0.51 | 0.62 | 1.79 | 0.76 | 0.59 |
| 2 | Minutes | | | | | |
| | No. | 135 | 87 | 17 | 16 | 255 |
| | % | 1.01 | 1.70 | 2.53 | 4.07 | 1.31 |
| 3 | Other bilat. | | | | | |
| | No. | 62 | 44 | 13 | 11 | 130 |
| | % | 0.47 | 0.86 | 1.94 | 2.80 | 0.67 |
| 4 | Unilat. | | | | | |
| | No. | 146 | 78 | 21 | 18 | 263 |
| | % | 1.10 | 1.52 | 3.13 | 4.58 | 1.35 |
| | Total | | | | | |
| | No. | 411 | 241 | 63 | 48 | 763 |
| | % | 3.1 | 4.7 | 9.4 | 12.2 | 3.91 |

The data on visible mutations with *res* also suggest relatively more chromosomal effect by the fast neutron treatment than by x-rays. These data are presented in tables 4, 5, and 6. Table 4 shows the frequency of the four classes of visibles in the fast neutron series. Table 5 shows the effects of several different dosages of soft x-rays. A comparison of the relative frequencies of the different types of visible mutations in the two kinds of radiated flies is given in table 6. The only class of visible mutations to show a statistically significant difference in relative frequency

is the class called "other bilaterals." They are relatively more frequent in the fast neutron series. This is shown in the summary in table 6 and can be seen by inspec-

TABLE 6

RELATIVE PERCENTAGES OF DIFFERENT CLASSES OF VISIBLE MUTATIONS IN TOTALS OF TABLES 4 AND 5

| TREATMENT | MUTANTS | CLASS 1 | 2 | 3 | 4 |
|---|---|---|---|---|---|
| Neutrons | 109 | 15 | 26 | 31 | 28 |
| X-rays | 763 | 15 | 33 | 17 | 35 |
| | Chi-square | 0.00 | 1.60 | 9.23 | 1.11 |

tion to be true in individual series in tables 4 and 5. This class of mutations consists mostly of dominant visible mutations which resemble well-known dominants found often in radiated material. Studied cytologically many of the previously found radiation-produced dominant visible mutations have proved to be associated

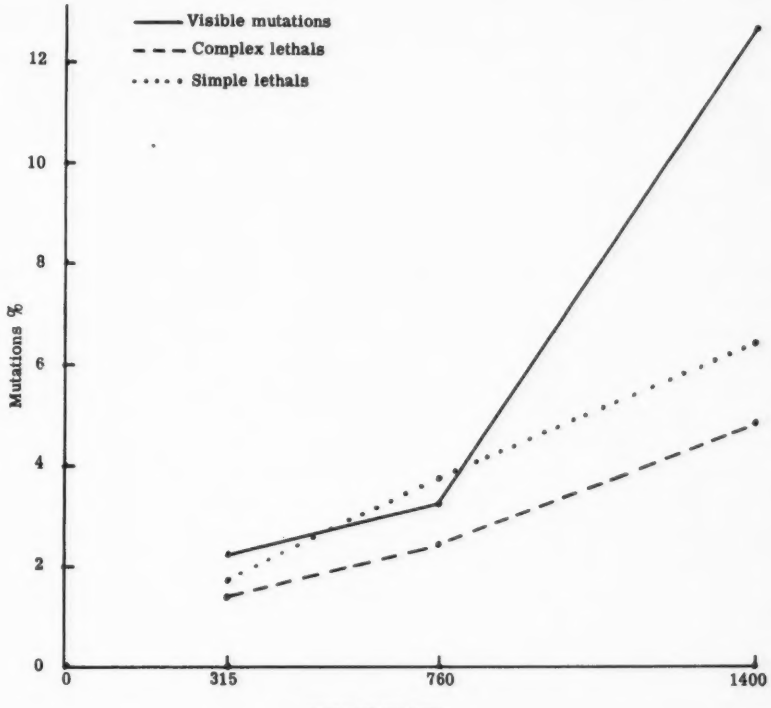


FIGURE 1

Dosage-mutation rate curves.

with easily detectable chromosome rearrangements which frequently give reduced crossing-over. It might be expected, therefore, that if fast neutrons produce relatively more gross chromosomal aberrations, this class of visibles would be increased relative to the other classes as in the present instance.

The data on autosomal visible mutation rates also indicate an over-all great effectiveness of fast neutrons. The total of 3.2 per cent visibles of all classes at 760 REP in table 4 is similar to the total of 3.1 per cent at 3000 r in table 5. The approximately four times greater effectiveness of the fast neutrons in producing autosomal visibles parallels the three and one-half times greater effectiveness in producing sex-linked lethal mutations.

Thus, it is evident that the data from autosomal visible mutations are in close agreement with those from sex-linked lethal mutations, both in the increased total mutation rate and in the increased portion of gross chromosomal aberrations in the fast neutron series.

Finally, it is possible to construct dosage curves for the three major classes of mutations observed in the fast neutron series: the simple lethals, the complex lethals, and the visible mutations of all kinds. This is done in figure 1. The slope of the curves for the two kinds of lethals is similar and the points approximate a straight line. The percentage of visible mutations, however, rose higher than expected in the 1400 REP series on such an interpretation. The data for this dosage are small (13 visibles in 103 test flies) and cannot be said to offer strong evidence against a straight line dosage curve, even though there is a P value of 1 per cent for the difference between 12.6 per cent and the average of the other two mutation rates at that dosage. Nevertheless, they do suggest that the curve may not be linear for visible mutations as a group.

The data on visibles are not large enough to warrant further breakdown into dosage curves for the several kinds of visibles. Further studies in this direction are in progress here involving soft x-rays, the gamma radiation of a 500 Curie Co-60 slug which is being installed at this laboratory, and both thermal and lower intensity fast neutrons now available elsewhere.

The finding of so much gross chromosome damage in lethals from the fast neutron radiation produced by an atomic explosion and the fact that the proportionate amount did not change as the frequency of the lethals rose with increasing dosage suggests that probably a single hit was able to produce two or more chromosome breaks. This appears to be characteristic of fast neutrons generally.[5, 6] It is not the case in the effects of x-rays where a single hit appears to be incapable generally of producing the two breaks necessary for a gross chromosome rearrangement.[7]

While the sex-linked lethal mutation rate in our x-ray series is comparable to that found generally with a similar dosage, the rates in our fast neutron series are comparatively higher than those found by others. Dempster,[5] Giles,[6] and others more recently have reported fast neutrons to be less effective than x-rays in producing sex-linked lethals in Drosophila. It seems probable that the reason for the opposite finding in our case is due to impoved methods now in use for estimating the REP dosage of fast neutrons. It needs to be emphasized therefore that fast neutrons, at least those produced by an atomic explosion, are much more effective than soft x-rays in producing sex-linked lethal mutations in *D. melanogaster*.

*Summary.*—The fast neutrons of an atomic explosion were three to four times as effective as x-rays in producing sex-linked lethal and autosomal visible mutations in the mature sperm of *D. melanogaster*. About 42 per cent of the sex-linked lethal chromosomes gave evidence of containing gross chromosomal aberrations, eight times as many as appeared in a group of mutator-caused lethals. The increase in

mutation rate with increasing dosage of fast neutrons appeared to be linear for sex-linked lethals, both with and without gross chromosomal aberrations; but the rate may not have been linear in the case of the autosomal visibles.

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[1] Alexander, Mary L., *Rec. Genetics Soc. Am.*, **21**, 7 (1952).

[2] Young, W. J., Yost, H. T., Jr., Ives, P. T., and Levine, R. P., these PROCEEDINGS, **39**, 488–495 (1953).

[3] Ives, P. T., *Evolution*, **4**, 236–252 (1950).

[4] Hinton, T., Ives, P. T., and Evans, Alice T., *Ibid.*, **6**, 19–28 (1952).

[5] Dempster, E. R., these PROCEEDINGS, **27**, 249–250 (1941).

[6] Giles, N. H., Jr., *Genetics*, **28**, 398–418 (1943).

[7] Lea, D. E., *Actions of Radiations on Living Cells*, Macmillan Co., New York, 1947, 402 pp.

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## *PROTECTIVE PROPERTIES OF CYSTEINE, SODIUM HYPOSULFITE, AND SODIUM CYANIDE AGAINST RADIATION INDUCED CHROMOSOME ABERRATIONS**

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*Introduction.*—Several investigators have shown the importance of the presence of oxygen to the process of chromosome breakage during exposures to x-rays. By lowering the oxygen tension below 2 per cent or by exposing tissue to x-rays in the presence of other gases such as nitrogen and helium, a considerable decrease in the frequencies of chromosome aberrations was obtained. This was shown by Thoday and Read[1] in the root tips of *Vicia faba*, by Hayden and Smith[2] in barley seeds, and by Giles and Riley[3, 4] in microspores of *Tradescantia paludosa*. By removal of most of the oxygen in the tissue the frequency of x-ray induced aberrations was reduced to nearly one-third of that obtained when the tissue was exposed in air or in higher oxygen tensions. Apparently, it is also possible to obtain a similar reduction in aberration frequencies with chemicals. Mikaelsen[5] has shown that reduced glutathione added to the nutrient solution of *Tradescantia paludosa* reduced the frequency of chromosome fragments by about 50 per cent after a 48-hour exposure to chronic $\gamma$-radiation at a dose rate of 25 r/day. The results obtained with glutathione suggested testing of other chemicals for protection against radiation induced chromosome aberrations. In the present paper the effect of cysteine, sodium hyposulfite, and sodium cyanide are reported.

*Material and Methods.*—Cuttings of *Tradescantia paludosa* (clone B2-2) were used as experimental material. Root development was initiated by placing the cuttings in tap water with continual aeration at ordinary greenhouse temperature (65–70°F.).[5] When the cuttings showed good development of primary roots, they were transferred to specially designed lucite vessels which contained Hoagland and Snyder's nutrient solution.[6] The vessels were 30 × 2 × 12 cm. in size. The side of the vessel facing the source was 3 mm. thick.

After 24 hours in the nutrient solution the cuttings were transferred to fresh nutrient solutions containing the various concentrations of chemical agents to be tested. After one hour, the plants, still in the solution of the chemical compound, were exposed to continuous $\gamma$-irradiation from a $CO^{60}$ source for 48 hours. In the experiments with cysteine-HCl and sodium hyposulfite§ a 3 curie $CO^{60}$ source was used. The source was located in a cylindrical, stainless steel container with a front wall thickness of 0.8mm. and 1 mm. thick side walls. This source holder was placed

TABLE 1

THE EFFECT OF DIFFERENT CONCENTRATIONS OF CYSTEINE, SODIUM HYPOSULFITE ($Na_2S_2O_4$) AND SODIUM CYANIDE (NaCN) ON THE FREQUENCIES OF CHROMOSOME FRAGMENTS AND BRIDGES DURING EXPOSURE TO $\gamma$-IRRADIATION

| CONCENTRATION USED | NO. OF CELLS SCORED | NO. OF FRAGMENTS | FRAGMENTS PER 100 Cells | PROTECTION, % | NO. OF BRIDGES | NO. OF BRIDGES PER 100 CELLS |
|---|---|---|---|---|---|---|
| | | (*a*) Cysteine-HCl (low pH) | | | | |
| None | 614 | 212 | 34.5 | 0 | 10 | 1.63 |
| $1 \times 10^{-5}$ *M* | 403 | 118 | 29.3 | 15 | 7 | 1.74 |
| $1 \times 10^{-4}$ *M* | 606 | 117 | 19.3 | 44 | 12 | 1.98 |
| $1 \times 10^{-3}$ *M* | 595 | 97 | 16.3 | 53 | 10 | 1.68 |
| | | (*b*) Cysteine-HCl (low pH) | | | | |
| None | 668 | 113 | 16.9 | 0 | 14 | 2.09 |
| $3 \times 10^{-4}$ *M* | 548 | 37 | 6.8 | 60 | 12 | 2.19 |
| $3 \times 10^{-3}$ *M* | Toxic | ... | ... | ... | ... | ... |
| $1 \times 10^{-2}$ *M* | Toxic | ... | ... | ... | ... | ... |
| | | (*c*) Cysteine-HCl (high pH) | | | | |
| None | 313 | 76 | 24.3 | 0 | 12 | 3.83 |
| $5 \times 10^{-4}$ *M* | 472 | 90 | 19.1 | 21 | 8 | 1.69 |
| $5 \times 10^{-3}$ *M* | 498 | 85 | 17.1 | 30 | 13 | 2.61 |
| $1 \times 10^{-2}$ *M* | 585 | 92 | 15.7 | 35 | 17 | 2.91 |
| | | (*d*) $Na_2S_2O_4$ | | | | |
| None | 141 | 33 | 23.4 | 0 | 5 | 3.55 |
| $1 \times 10^{-5}$ *M* | 149 | 19 | 12.8 | 46 | 5 | 3.36 |
| $1 \times 10^{-4}$ *M* | 211 | 29 | 13.7 | 41 | 5 | 2.29 |
| $1 \times 10^{-3}$ *M* | Toxic | ... | ... | ... | ... | ... |
| | | (*e*) NaCN | | | | |
| None | 569 | 105 | 18.5 | 0 | 19 | 3.34 |
| $1 \times 10^{-4}$ *M* | 696 | 115 | 16.5 | 11 | 20 | 2.87 |
| $5 \times 10^{-4}$ *M* | 103 | 17 | 16.5 | 11 | 5 | 4.85 |
| | | (*f*) NaCN | | | | |
| None | 239 | 54 | 22.6 | 0 | 19 | 3.77 |
| $1 \times 10^{-3}$ *M* | 178 | 30 | 16.9 | 25 | 4 | 2.25 |

in the center of a spherical lead pig with a cone-shaped opening of about 70° for the beam. In the experiments with sodium cyanide an 8.5 curie $CO^{60}$ source was used. The source was located in a similar source holder and was placed in a cylindrical aluminum pipe with a wall 1 mm. thick. The pipe was located near the corner of an L-shaped lead and concrete shield, which gave an irradiation field of about 90°. Dosages, as measured by the film badge method,[7] indicated that the plants in all the experiments received approximately 25 r/day and hence a total dose of 50 r.

Immediately following the 48-hour exposure period the root tips were fixed in alcohol-glacial acetic acid (3:1). Slides were prepared using the propionic- and aceto-carmine squash technique.

In the experiments reported below the following chemicals were tested: cysteine-HCl, sodium hyposulfite ($Na_2S_2O_4$), a strong reducing agent, and sodium cyanide (NaCN).

The number of both acentric fragments and bridges per 100 cells in anaphase of meristematic root tip cells was determined. However, conclusions regarding the effects of chemical treatments were based upon the fragment data only, since bridges occurred in such low frequency that it was difficult to evaluate the data.

*The Influence of Various Chemicals on the Frequencies of Chromosome Fragmentation.*—The frequencies of acentric fragments in the untreated control series exposed to chronic $\gamma$-irradiation were about the same in 4 of 5 experiments and varied from 16.9 to 24.3 per cent (table 1 (*b*), (*c*), (*d*), (*e*), (*f*)). However, the control series in the remaining experiment had a much higher number of fragments (34.5 per cent, table 1 (*a*)). The reason for this high value is not clear.

(*a*) *Cysteine Hydrochloride (Low pH)*: Cysteine-HCl was applied in two different experiments (table 1 (*a*) and (*b*)). In the first experiment (table 1 (*a*)) the following concentrations of cysteine-HCl were used: $10^{-5}$ *M*, $10^{-4}$ *M*, and $10^{-3}$ *M*. The presence of cysteine-HCl lowered the pH of the nutrient solution in which the plants were grown, particularly with the two strongest concentrations. The pH of the one-day old nutrient solution in which the plants were kept was about 5 just before the irradiation exposure started. After 48 hours the pH of the solution increased to about 6.6. In $10^{-5}$ *M* cysteine-HCl the pH was slightly lower, 4.5 and 6.2, respectively, before and after the experiments. At the beginning of the experiment the pH was 2.5 and 3, respectively, for the stronger concentrations, i.e., $10^{-3}$ *M* and $10^{-4}$ *M*. By the end of the experiment the pH values had increased to 4.2 and 4.6, respectively, for the same concentrations. Thus, the plants were grown and exposed to cysteine under quite different pH values. Judging by mitotic activity, the different pH values did not seriously disturb the roots since equal numbers of dividing cells were present in all cases.

In the first experiment the control series gave a fragment frequency of 34.5 per 100 anaphase cells after chronic $\gamma$-irradiation. In the presence of cysteine at a concentration of $10^{-5}$ *M* the number of fragments decreased slightly to 29.3 per 100 cells. Further decrease in fragment frequency was obtained with increasing cysteine concentrations. Treatments with $10^{-4}$ *M* and $10^{-3}$ *M* solutions gave, respectively, 19.3 and 16.3 fragments per 100 cells. The latter was a decrease of 53 per cent over that obtained in the control, which was the maximum effect in that experiment.

In another experiment the concentrations $3 \times 10^{-4}$ *M*, $3 \times 10^{-3}$ *M*, and $10^{-2}$ *M* were tested. Both $3 \times 10^{-3}$ *M* and $10^{-2}$ *M* proved to be toxic to the roots. The roots became soft and the cells were damaged and unsuitable for studies. In the $3 \times 10^{-4}$ *M* cysteine-HCl solution, however, the number of fragments per 100 cells was reduced from 16.9 which was obtained in the control to 6.8. This is a reduction in fragment frequencies of 60 per cent, which is the maximum effect obtained with cysteine. In figure 1 the results from these two experiments are plotted in curve *A* on a semilogarithmic scale. (*A* linear relation between protective ability and concentration is suggested.)

(*b*) *Cysteine-HCl in Partly Neutralized Medium of pH ~ 6:* At the beginning of another experiment where cysteine-HCl was used, each cysteine solution was adjusted to a pH of approximately 6 by the addition of potassium hydroxide. This was also the pH of the nutrient solution. Cysteine hydrochloride was used in concentrations of $5 \times 10^{-4}$, $5 \times 10^{-3}$ and $10^{-2}$ *M*. Thus, in this experiment all the mixtures, those containing cysteine and the control, were exposed to irradiation at the same pH value. The last two concentrations were stronger than those previously employed. In this experiment (table 1 (*c*)) the fragment frequency fell from 24.3 per 100 cells in the control to 15.7 with $10^{-2}$ *M* cysteine, which was the

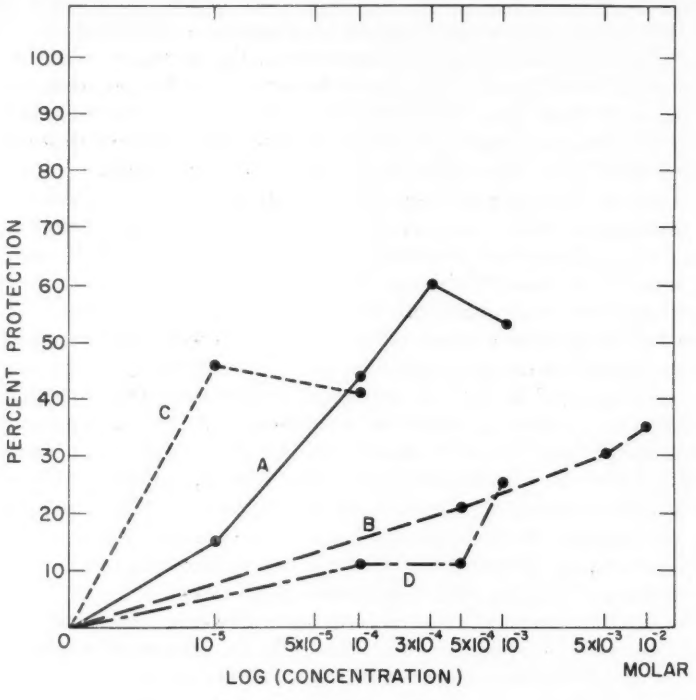


FIGURE 1

Relationship between concentration of chemicals used and percentage of protection. *A*, Cysteine, low pH. *B*, Cysteine, high pH. *C*, Sodium hyposulfite. *D*, Sodium cyanide.

strongest concentration used in the experiment. The intermediate concentrations, $5 \times 10^{-4}$ and $5 \times 10^{-3}$, yielded fragment frequencies of 19.1 and 17.1, respectively, per 100 cells. The relation between per cent protection and concentration is plotted on a semilogarithmic scale (Fig. 1 (*B*)). There is an almost straight line relationship.

The results (table 1 (*c*)) demonstrate very clearly that cysteine in a partly "neutralized" medium (pH approx. 6) gave considerably less protection to the chromosomes than was obtained in the experiments with cysteine at lower pH-values (table 1 (*a*), (*b*)). The greatest effect of the neutralized cysteine was a 35 per cent reduction in fragment frequency, compared to the 60 per cent reduction of the pre-

vious cysteine experiments. The graphs in figure 1 clearly demonstrate this difference.

(*c*) *The Effect of Sodium Hyposulfite, $Na_2S_2O_4$:* Sodium hyposulfite was applied in the same manner as cysteine-HCl. Because $Na_2S_2O_4$ is easily oxidized, a fresh solution was substituted after the first 24 hours' exposure to radiation. As can be seen from table 1 (*d*) and figure 1 (*C*), a remarkable and significant effect was obtained even at the low concentration of $10^{-5}$ *M*. This series gave 12.8 fragments per 100 cells as compared to 23.4 in the control. At the stronger concentration, $10^{-4}$ *M*, no further decrease was obtained in the fragment frequency, 13.7 being insignificantly higher than 12.8 obtained at the previous concentration. A concentration of $10^{-3}$ *M* was also used, but appeared to be toxic to the roots. Thus, the maximum protection obtained with sodium hyposulfite was 46 per cent.

(*d*) *The Effect of Sodium Cyanide, NaCN:* The results with sodium cyanide, NaCN, used in two different experiments are presented in table 1 (*e*) and (*f*). Concentrations of $10^{-4}$ *M* and $.5 \times 10^{-4}$ *M* both gave 16.5 fragments per 100 cells as compared to 18.5 in the untreated control series. Thus, sodium cyanide afforded a relatively small amount of protection (about 10 per cent) at these concentrations. In another experiment (table 1 (*f*))at a concentration of $10^{-3}$ *M* a marked decrease in number of fragments was obtained. The fragment frequency was reduced from 22.6 per 100 cells in the control to 16.9 in the NaCN series. This significant reduction amounts to a protection of 25 per cent.

The effects of increased concentrations of sodium cyanide are also presented in figure 1 (*D*).

*The Occurrence of Chromosome Bridges.*—As previously indicated,[5] chromosome or chromatid reunions resulting in bridge formations at anaphase were infrequent in root tip cells following exposures to chronic irradiation. Due to a similar low frequency of bridges in the present experiments no definite conclusions can be drawn. More data relating to rejoining are required.

*Discussion.*—Conclusive evidence is presented for the protective ability of cysteine, sodium hyposulfite ($Na_2S_2O_4$), and sodium cyanide (NaCN) against chromosome fragmentation induced by irradiation. The mechanism, however, by which these chemicals exert their protection is not too clear. Sparrow[8] and Sparrow and Rubin[9] have discussed several factors which influence the radiosensitivity of chromosomes. Various agents which modify the genetic and cytological effects of ionizing radiations are also emphasized and the importance of an indirect radiation effect is considered. Mikaelsen[5] has emphasized the possibilities that the role of glutathione is through reduced chromosome breakage or by increased restitution of broken ends. A combination of both is a third possibility. The prevention of chromosome breakage was considered the most likely explanation.

Since the completion of these experiments Forssberg and Nybom[13] have also demonstrated a reduction in chromosome disturbances produced by x-radiation in the presence of cysteine in *Allium* roots. However, Devik[14] found no effect on chromosomal aberrations following treatments of mice with cysteine prior to x-ray exposures.

The effect of cysteine appeared to be very different in an acid and a neutral medium (table 1, Fig. 1). A similar response in the effect of cysteine, applied at different pH values, on survival of mice after whole body x-irradiation was ob-

tained by Patt, *et al.*,[15, 16] and Goldie, *et al.*[17] They state that the probable explanation of this difference may be the fairly rapid oxidation of cysteine to cystine in a neutral medium before its administration. It has been demonstrated that changes in intracellular acidity alter the radiosensitivity of the cell.[18–21] It seems justifiable, however, in this case to put the main emphasis on the oxidation process of cysteine to cystine.

Weiss,[10] Lea,[11] and others state that during exposures of water to ionizing radiation, oxidizing agents such as $H_2O_2$, O and the radicals OH and $O_2H$ are formed. Since water exists in the cell nuclei, it is likely that the same reactive agents are formed in the nuclei during exposures to ionizing radiation. Barron and Flood[12] presume that these oxidizing agents are responsible for the oxidation of aqueous solutions of certain thiols by ionizing radiation, as they have demonstrated. Therefore, it seems probable that the mechanism of the sulfhydryl compound, cysteine as well as glutathione,[5] may be due to oxidizing agents produced by irradiation. These oxidizing agents may not necessarily be those mentioned above. The formation of other reactive agents (i.e., organic free radicals or organic peroxides) must also be considered.

The mechanism of the effect of sodium hyposulfite in reducing fragment frequencies is probably different from that of sulfhydryl compounds. Since sodium hyposulfite is a strong reducing agent and combines rapidly with molecular oxygen in aqueous solution, it is reasonable to assume that sodium hyposulfite decreases the amount of dissolved oxygen in the tissue. Burnett, *et al.*,[22] explained the increased survival in bacteria suspension in the presence of sodium hyposulfite during the irradiation exposure to be due to the removal of oxygen from the suspensions. By removing oxygen from the tissue, the diversion of H, for example, into the production of the secondary formation of $O_2H$ and $H_2O_2$ or other reactive products is prevented. Thus, less radiochemical reactants will be formed and less breaks will be obtained. King, *et al.*,[23] claim, however, that in a large measure oxygen exerts its action by altering the biochemical processes of the cell.

In the presence of sodium hyposulfite the fragment frequency was reduced to almost half. A similar response in effect of sodium hyposulfite is obtained by Riley[24] in *Allium* roots after exposure to acute $\gamma$-irradiation. Giles and Riley[3, 4] were able to reduce the aberration frequency to one-third in Tradescantia, when exposed in vacuum or an inert gas. The difference between the effect of low-oxygen tension and sodium hyposulfite may probably be due to an incomplete removal of ozygen by sodium hyposulfite rather than to a different mechanism.

The protective ability of sodium cyanide may be more complicated and obscure than was the case with the sulfhydryl compounds and sodium hyposulfite. Bacq[25] claimed that the $CN^-$ was responsible for the decrease in mortality that was obtained with cyanide in x-irradiated mice; the mechanism by which $CN^-$ exerts its effect, however, is not understood. It appears, especially, as regards the two strongest concentrations, that cyanides reduce the mitotic rate. Considerably fewer anaphases (table 1) were found in these roots and the reduction in fragment frequency was small compared to the control series and the concurrent experiments with the other chemical agents. These facts indicate that metabolic inhibition or metabolic changes may be the most reasonable explanation of the protective effect of sodium cyanide. Such metabolic alterations initiated by KCN and CO are dis-

cussed by King, *et al.*,[23] and complicated enzyme systems are probably involved.

D'Amato and Gustafsson[26] have shown that cyanide (KCN) treatment of seeds increased visible mutations in barley with low concentrations of KCN. A stronger concentration $10^{-2}$ *M*, decreased the mutation rate, although the rate of chromosome breakage increased. These results may not be comparable to the results presented in this paper, but may indicate the complicated effects of cyanides.

Although the protective actions of these chemicals is not fully understood, evidence of an indirect effect of radiation on chromosome fragmentation is demonstrated. This indirect effect seems to be of great importance, since it is possible to reduce the radiation effect to about half in some cases (cysteine, at low pH, $Na_2S_2O_4$, glutathione[5]). Unfortunately, however, it will be difficult to determine the exact relation between direct and indirect radiation effect by chemical means, because of their toxic effects to roots at certain concentrations (table 1).

*Summary.*—The protective effect of cysteine, sodium hyposulfite ($Na_2S_2O_4$) and sodium cyanide against chromosome fragmentation induced by $\gamma$-irradiation is demonstrated.

The maximum effect of cysteine applied as cysteine-HCl, is a reduction in number of fragments of 60 per cent at $3 \times 10^{-4}$ *M* (low pH). With "neutralized" cysteine-HCl (pH $\sim$ 6) the maximum reduction amounts only to 35 per cent at $10^{-2}$ *M*. The difference in protective ability between cysteine at low and high pH, is thought to be due to easy decomposition of cysteine to cystine in a neutralized medium.

The protective action of cysteine in the cell nuclei is explained by its possible reactions with oxidizing agents produced in the nuclei by irradiation.

Sodium hyposulfite ($Na_2S_2O_4$) reduces the fragment frequency 46 per cent at $10^{-5}$ *M*. As a strong reducing agent, it is suggested that its main effect is removal of oxygen from the tissue and that this interferes with the formation of certain reactants normally produced during irradiation in the presence of oxygen.

Sodium cyanide at $10^{-3}$ *M* also shows a marked protective effect and decreases the number of fragments by 25 per cent. However, the mechanism of the protective action of NaCN seems more obscure than that of the other chemical agents tested in these experiments. Induced metabolic changes may be involved.

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§ Also called hydrosulfite. Not to be confused with the thiosulfate ($Na_2S_2O_3$) which is also called hyposulfite.

[1] Thoday, J. M., and Read, J., *Nature*, **160**, 608–610 (1947).

[2] Hayden, B., and Smith, L., *Genetics*, **34**, 26–43 (1949).

[3] Giles, N. H., Jr., and Riley, H. P., PROC. NATL. ACAD. SCI., **35**, 640–646 (1949).

[4] Giles, N. H., Jr., and Riley, H. P., *Ibid.*, **36**, 337–344 (1950).
[5] Mikaelsen, K., *Science*, **116**, 172–174 (1952).
[6] Hoagland, D. R., and Snyder, W. C., *Proc. Am. Soc. Hort. Sci.*, **30**, 288–294 (1933).
[7] Ehrlich, M., and Fitch, S. H., *Nucleonics*, **9** (3), 5–17 (1951).
[8] Sparrow, A. H., *Ann. N. Y. Acad. Sci.*, **51**, 1508–1540 (1951).
[9] Sparrow, A. H., and Rubin, B. A., *Survey Biol. Progr.* **2**, 1–52 (1952).
[10] Weiss, J., *Brit. J. Radiol. Suppl.*, **1**, 56–59 (1947).
[11] Lea, D. E., *Ibid.*, **1**, 59–63 (1947).
[12] Barron, E. S., and Flood, V., *J. Gen. Physiol.*, **33**, 229–241 (1950).
[13] Forssberg, A., and Nybom, N., *Physiol. Plant.*, **6**, 78–95 (1953).
[14] Devik, F., *Brit. J. Radiol.*, **27**, 481–484 (1952).
[15] Patt, H. M., Tyree, E. B., Straube, R. L., and Smith, D. E., *Science*, **110**, 213–214 (1949).
[16] Patt, H. M., Smith, D. E., Tyree, E. B., and Straube, R. L., *Proc. Soc. Exptl. Biol. Med.*, **73**, 18–21 (1950).
[17] Goldie, H., Tarleton, G. T., Jr., and Hahn, P. F., *Ibid.*, **77**, 790–794 (1951).
[18] Zirkle, R. E., *Am. J. Roentgenol.*, **35**, 230–237 (1936).
[19] Zirkle, R. E., *J. Cell. Comp. Physiol.*, **16**, 301–311 (1940).
[20] Zirkle, R. E., *Ibid.*, **17**, 65–70 (1941).
[21] Marshak, A., *Proc. Soc. Exptl. Biol. Med.*, **39**, 194–198 (1938).
[22] Burnett, W. T., Jr., Stapleton, G. E., Morse, M. L., and Hollaender, A., *Ibid.*, **77**, 636–638 (1951).
[23] King, E., Schneiderman, H., and Sax, K., PROC. NATL. ACAD. SCI., **38**, 34–43 (1952).
[24] Riley, H. P., *Rec., Genetics Soc. Am.*, No. 21, 62–63 (1952).
[25] Bacq, Z. M., *Science*, **111**, 356 (1950).
[26] D'Amato, F., and Gustafsson, A., *Hereditas*, **34**, 181–192 (1948).

## *THE DELAYED APPEARANCE OF MUTANTS IN BACTERIAL CULTURES**

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In the course of an examination of the possibility of a delay in the growth of mutant clones because of the need for segregation of mutant from non-mutant nuclei,[1] experiments of the following sort were carried out. A high proportion of new mutants was produced by the use of ultra-violet light. The irradiated culture was spread on the surfaces of several plates consisting of an agar medium on which only mutants could form colonies. At various times thereafter the surfaces of some of the plates were respread. The number of colonies resulting would be expected to increase only if the spreading occurred after the newly arisen mutant nuclei had been separated by cell division from the non-mutated nuclei in the originally multinucleate bacteria. The delay in onset of division of the new mutants was found to be far too long to be accounted for by the hypothesis of nuclear segregation. In fact in some cases it was more than twenty-five times as long as that of the parental bacteria and of old mutants which had been irradiated and mixed on the same plates. The differential sensitivity of the new mutants requires explanation and has a direct bearing on the problem of the delayed appearance of mutants that has been observed by others.[2]

The experimental conditions were as follows. The strains used were all derived from *Escherichia coli* 15; $h^-lac^+$ has been previously described;[1] $h^-lac^-$, which not only is unable to synthesize histidine but cannot ferment lactose, was secured from $h^-lac^+$ after treatment with ultra-violet light; $h^+lac^+$ and $h^+lac^-$ were isolated after spontaneous mutation of the $h^-$ character in the appropriate strains. All cultures were grown in minimal medium supplemented with histidine as described elsewhere.[1] For the irradiation, 0.1 ml. of a fully grown culture was inoculated into 200 ml. of minimal medium with histidine (25$\mu$g. per ml.) and a limiting amount (0.05 per cent) of glucose and shaken at 37°C. for about 12 hours. The cultures were then centrifuged, resuspended in one-tenth of the original volume of saline and distributed to Petri dishes in 5-ml. lots. These were illuminated for 120 seconds while being rotated at a distance of 14 cm. from a Westinghouse Sterilamp. The suspensions were then pooled, washed once in saline, and resuspended so as to make a total concentration of one-hundred fold. They were then spread as 0.2-ml. aliquots on the surface of agar plates devoid of histidine. All operations from the irradiation through the incubation of the plates were performed in the yellow light of a G.E. Bug-a-way lamp to prevent photoreactivation. There was no demonstrable difference between the four strains in their sensitivity to radiation, a finding confirming that of Lieb.[3] The irradiation dose used resulted in a survival of *ca.* $10^{-6}$ bacteria and caused the appearance of *ca.* $10^{-3}$ $h^+$ mutants among the survivors, whereas the background frequency of $h^+$ mutants in $h^-$ cultures was about $10^{-7}$. This high frequency of induced $h^+$ mutants confirms the work of Kaplan[4] but is in contradiction to the claims of Lieb.[3] In cases of high killing the induced mutant colonies do not appear until after three days of incubation, and thereafter their number does not increase significantly; it is probable that Lieb's plates were counted too early. The curve for the increase in the proportion of mutants among the survivors was linear when the logarithm of the proportion was plotted against dose. This held true even over regions of dose where a semilogarithmic plot of survivors showed a break indicating the appearance of an ultra-violet resistant fraction in the bacterial population.

The plates used in these experiments consisted of minimal medium solidified with 2 per cent agar. They were dried by storage at 37°C. for two days before use. The agar had been carefully washed in order to prevent residual growth by the $h^-$ bacteria; these bacteria did not grow or form colonies unless histidine was added. To all plates 0.2 ml. of a concentrated irradiated culture of either $h^-lac^+$ or $h^-lac^-$ bacteria was added and immediately spread by the use of a sterile glass rod and a rotating stand. The droplet of bacterial suspension was absorbed by the agar during this procedure. To half of the $h^-lac^+$ plates 0.5 ml. of a $10^{-3}$ dilution of a culture of $h^+lac^-$ bacteria, irradiated to the same extent and exhibiting the same survival, was also added; to half of the $h^-lac^-$ plates similarly treated $h^+lac^+$ bacteria were added. The experiment was done in this reciprocal way in order to reveal any possible effect of the lac marker on the outcome. All plates were incubated at 37°C. and removed only for the times required to respread the surfaces. This was achieved by adding 0.2 ml. of sterile saline in which the surfaces were again scrubbed by the use of a glass rod and turntable. After respreading, the plates were reincubated for three days when the colonies were counted. The $h^+$ colonies present were isolated to Endo agar where their ability to ferment lactose was determined.

Figure 1 summarizes the results obtained. The rising curve shows the growth of the added $h^+$ bacteria which in six experiments had an average lag period of about one and one-half hours and an average generation time of 36 minutes. This normal behavior demonstrates two things. First, the spreading technique adequately separated the components of the $h^+$ micro-colonies formed by growth on the agar. Second, the old $h^+$ bacteria that survived the irradiation were not significantly injured by it.[5]

On the other hand, $h^+$ mutants newly derived from the $h^-$ parents as a result of irradiation exhibit a variable and sometimes extremely long delay in the onset of growth. Growth in some cases occurred within five hours of irradiation; in other instances it did not commence until sometime after 27 hours. The average length of the delay *per bacterium* must be even longer for there were *ca.* 10 $h^+$ mutants per plate; the initiation of growth by any one of them would increase the number of

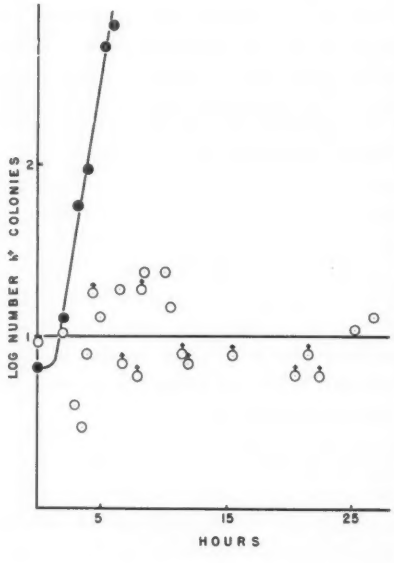


FIGURE 1

The increase in numbers of $h^+$ colonies on plates devoid of histidine to which cultures irradiated for 120 seconds had been added and respread. The ordinate measures the $\log_{10}$ of the number of colonies found after three days and the abscissa, the number of hours after the original spreading at which the respreading was performed. The ascending curve with black circles is for the average number of colonies of added $h^+lac^-$ and $h^+lac^+$ bacteria. The generation time was 36 minutes and the lag period about one and one-half hours. The open circles are the average number of colonies of $h^+lac^+$ and $h^+lac^-$ mutants arising among $h^-lac^+$ or $h^-lac^-$ bacteria which had been introduced onto the surface of the plates by themselves or with $h^+$ bacteria of the opposite lac type. The arrows indicate that one plate of a pair had supported enough growth at the time of respreading so that the resulting colonies were too numerous to count. The black circles come from six separate experiments; the open circles from the same experiments plus an additional seven.

colonies on the plates. The difference between old and newly arisen $h^+$ bacteria was not a function of the lac marker used; results were the same with both combinations.

A delay of 27 hours, if explained on the hypothesis of segregation lag, would require the separation of one from more than $10^{13}$ mutable units. For this reason the hypothesis was rejected. The delay is, of course, a species of phenotypic lag but of a different sort than that discussed in the literature.[6, 7] The newly arisen mutants do not express their $h^+$ character until they grow but the delay is more than that *required* to express that character. In other experiments, where $h^-lac^+$ bacteria were exposed to ultra-violet light for only 20 seconds and where *ca.* $10^{-3}$ of the bacteria survived, the new mutants which constituted *ca.* 99 per cent of the total

present showed little if any difference in lag from old mutants (Fig. 2). Other experiments (unpublished results; Lieb[3]) confirm these findings and indicate that, if ordinary phenotypic lag in the expression of the $h^+$ character occurs, it is not longer than one generation.

Nevertheless, if the expression of the phenotype did require at least a generation of growth, this could possibly occur on the plates through the provision of some histidine by the autolysis of the many dead bacteria present. Thus, the long delay in the onset of growth of the mutants could be due to the very slow growth of bacteria genotypically mutant but requiring at least a generation for the expression of their mutant character. This possibility was examined in the following experiment. Bacteria irradiated for 120 seconds were removed at intervals from the surfaces of plates devoid of histidine on which they had been spread. Removal was

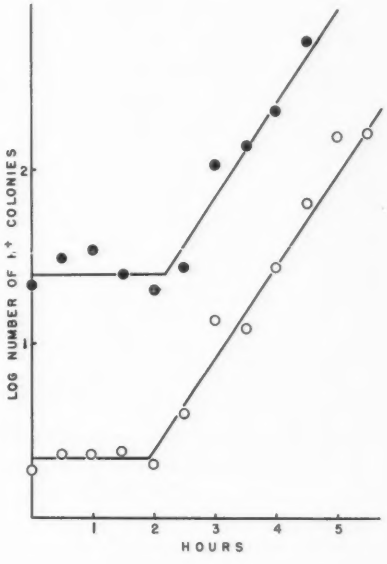


FIGURE 2

The increase in numbers of $h^+$ colonies on plates devoid of histidine to which cultures irradiated for 20 seconds had been added and respread. The ordinate measures $\log_{10}$ of the number of colonies found after three days and the abscissa, the number of hours after the original spreading at which respreading was performed. The black circles are for the average numbers of colonies of $h^+lac^-$ bacteria which had been mixed with $h^-lac^+$ organisms; the growth of $h^+lac^+$ mutants arising among the latter is shown by the open circles. The generation times were 34 minutes and the lag periods about two hours. The data come from two separate experiments in which triplicate plates were used.

achieved by the addition of 5 ml. of saline in which the surfaces were scrubbed with the aid of a glass rod and turntable; the suspension so formed was removed and the surface scrubbed again in another 5 ml. of saline. The two suspensions were pooled, washed, resuspended in 1 ml. of saline, appropriately diluted and plated on agar with and without histidine. The results are shown in figure 3. There is apparently a good deal of histidine available from the dead bacteria present. The viable $h^-$ bacteria began to grow after a lag period of about three hours and, with a generation time of *ca.* 60 minutes, increased to a final number of *ca.* $4 \times 10^7$. This level is equivalent to less than 1 $\mu$g. of histidine per plate which was provided by some $10^{10}$ killed bacteria; the washed agar provided less than $10^{-3}$ of this or less than that required for the growth of $10^4$ $h^-$ bacteria. (The number of $h^+$ mutants produced spontaneously at a rate of *ca.* $3 \times 10^{-8}$ per bacterium per generation during this $h^-$ growth is small (*ca.* 1) compared to the number of mutants present

that were induced by ultra-violet light (*ca.* 14)). Despite the almost normal growth of the $h^-$ bacteria, the $h^+$ bacteria underwent a lag of some 10 to 17 generations. A bacterium which is genotypically $h^+$ but phenotypically $h^-$ would not be expected to grow very slowly on these plates, but nevertheless there is a lag induced in the $h^+$ mutants which is considerably greater than that undergone by the $h^-$.

The fact that the delay in the onset of division of the new $h^+$ mutants is independent of the necessity to synthesize their own histidine is shown definitively by the next experiment. After being irradiated for 120 seconds, $h^-$ bacteria were washed and spread on plates *containing* histidine. At various times thereafter they were removed and plated by the method just described. The results are

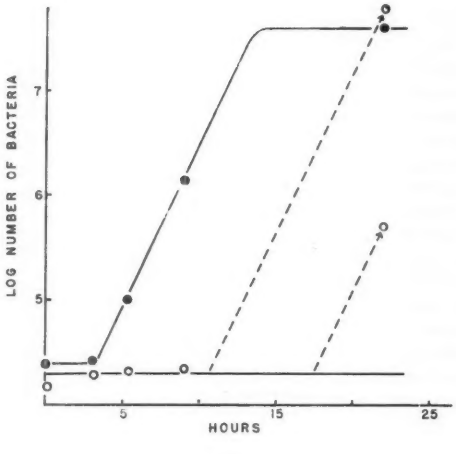


FIGURE 3

The increase in numbers of $h^+$ and $h^-$ bacteria on plates devoid of histidine to which $h^-$ cultures irradiated for 120 seconds had been added. The ordinate measures the $\log_{10}$ of the number of bacteria per plate and the abscissa, the number of hours after spreading at which the bacteria were removed from the plates. The black circles are for the numbers of $h^-$ bacteria and the open circles for the $h^+$ mutants that arose among them. In order to plot the numbers of $h^+$ bacteria on the same scale as the $h^-$ bacteria the numbers of the former have been multiplied by $10^3$. The generation time for the $h^-$ bacteria was about 60 minutes and the lag period about three hours. The data came from two experiments in which duplicate plates were used. The two points for $h^+$ at 22 hours have been plotted separately and not averaged because of the great difference in numbers they represent.

shown in figure 4. The $h^-$ bacteria increased normally with a generation time of 40 minutes and a lag period of about two hours.[5] Once again the $h^+$ mutants failed to increase at least for more than 8 hours, or for as may as 9 generations after their parents had begun to divide. In two experiments of this sort the plates were incubated for 24 hours before the bacteria were assayed. Due to the presence of added histidine and products provided by the dead bacteria the $h^-$ population on the plate had reached a level of *ca.* $2 \times 10^{10}$. By this time the $h^+$ mutants had also grown some; but their frequency was only $2 \times 10^{-6}$, while it was $10^{-3}$ immediately after irradiation when they were first spread on the plates. This long delay in the onset of division of new $h^+$ mutants could not be due to the requirement for processes leading to the $h^+$ phenotype since this phenotype is not required for growth in histidine.

These considerations force the conclusion that newly arisen $h^+$ mutants are different from old $h^+$ mutants and their $h^-$ parents in that high doses of ultra-violet light induced a severe delay in the onset of growth of the former. This special sensitivity could hardly have been predicted from what knowledge is available of the difference between $h^+$ and $h^-$ bacteria. It is not known whether $h^-$ bacteria actually lack histidine synthesizing enzymes or even whether $h^+$ bacteria when growing on histidine synthesize these enzymes. It is only known that $h^-$ bacteria cannot synthesize histidine and that biochemical pathways around the genetic block are probably disturbed.

On the other hand, it may be that most of the $h^+$ bacteria arising after high doses of ultra-violet light are mutant for the same reason that they are unable to initiate growth immediately after irradiation. The ultra-violet light may induce some fundamental change in a fraction of the bacteria; one result of the alteration

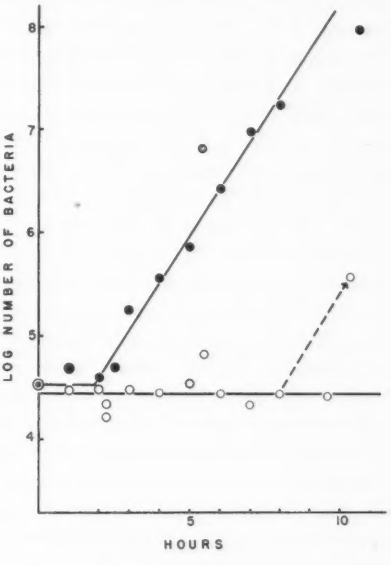


FIGURE 4

The increase in numbers of $h^+$ and $h^-$ bacteria on plates containing histidine to which $h^-$ cultures irradiated for 120 seconds had been added. The ordinate measures the $\log_{10}$ of the number of bacteria per plate and the abscissa, the number of hours after spreading at which the bacteria were removed from the plates. The black circles are for the numbers of $h^-$ bacteria and the open circles for the $h^+$ mutants that arose among them. In order to plot the numbers of $h^+$ bacteria on the same scale as the $h^-$ bacteri- the numbers of the former have been multiplied by $10^3$. The generation time for the $h^-$ bacteria was 40 minutes and the lag period about two hours. The data came from four separate experiments in which triplicate plates were used.

may be that the likelihood of a genetic change at the h locus is increased (there is no prejudice here as to whether this locus is chromosomal or otherwise). If the change is severe enough after high doses of ultra-violet light, it may result in a delay in the onset of growth and at the same time even further increase the chance of mutation. Since mutations occur after low doses of ultra-violet light which do not induce a delay, the delay cannot be the *cause* of mutation. But both may be caused by a common denominator induced by the radiation. This interpretation has the advantage of being based upon the numerous evidences which indicate that the mutagenic effect of ultra-violet light may be indirect, and of not requiring the assumption of unsuspected differences between new and old $h^+$ bacteria.

A correlation between delay in onset of division and mutation implies that mutations are correlated with one another. This has in fact been found by Witkin[8] to be the case for ultra-violet-induced mutations to lactose non-fermentation,

streptomycin independence, and phage $T_1$ resistance in *E. coli*. Her results were not confirmed by Newcombe[2] nor have different spontaneous mutations in our experience or in that of Newcombe and Hawirko[9] shown any correlation with one another. However, mutations to phage resistance induced by ultra-violet and by manganese have frequently been found to be associated with auxotrophic mutations.[10] The subject obviously requires further investigation, but it is clear, nevertheless, from the results shown in figures 1, 3, and 4, that newly arisen $h^+$ mutants induced by high doses of ultra-violet light are specifically delayed in growth. This is true whether the delay is associated with the cause or is an effect of mutation.

These experiments do not conclusively demonstrate that the induced $h^+$ mutants are "zero point"[11] mutants which make their appearance without preliminary incubation. Although the number of mutants did not increase for long periods of time on the plates where histidine was present, this may have been despite the division of bacteria genotypically $h^+$ in only one nucleus. A hidden division could have been responsible for the expression of the $h^+$ character. On the other hand the simpler hypothesis, that the $h^+$ bacteria did not multiply during the period when their numbers did not increase but nevertheless expressed their phenotypes, is equally possible. Were the latter true the $h^+$ character would have to be expressed in multinucleate bacteria where only one nucleus of several had mutated to the $h^+$ condition.[1] Hence that character would be "dominant." This is what one would expect by analogy with the behavior of growth factor synthesizing characters in sexually reproducing *E. coli*.[12] It is clear that the $h^+$ character requires that there intervene, between it and the mutational event, a synthesis, or at least an activation,[13] of enzyme used in the manufacture of histidine. This process might take place, however, without growth and in less time than is required for a generation.

Induced phage-resistant mutants, on the other hand, require preliminary cultivation for expression and do not appear as "zero point" mutants. Older claims to the contrary[11] were based upon experiments in which it was later discovered that the treated bacteria had divided before they were attacked by phage.[2, 14] If phage resistance is a recessive character, as it is found to be in sexually reproducing *E. coli*,[12] then, in multinucleate bacteria where a mutation has occurred in one nucleus, that nucleus must segregate from its sisters before it can impose a mutant phenotype on the cell. The delay required for the formation of a cell homocaryotic for mutant nuclei is referred to as segregation lag; before segregation lag is over the heterocaryotic mutant cell has the non-mutant phenotype and can be lysed by phage. Thus, it is clear that no "zero point" mutants would be expected from multinucleate cells when the character is recessive. When bacteria possess only one nucleus,[8] or, more strictly, when there is only one mutable unit in each uninucleate cell, the absence of "zero point" mutants may speak for the existence of a delay in the expression of the phenotype which, in the case of phage resistance, involves a reorganization of the cell surface.[15] In such systems, where there are no "zero point" mutants but where there is a phenotypic or segregation lag of a generation or more, an induced delay in the growth of new mutants would have serious effects.

For example, Demerec[11, 16] showed that the full complement of radiation-induced mutants resistant to phage $T_1$ was not expressed until after about 13 generations of

growth by the parent population of *E. coli*. Were the mutants subject to an induced delay in growth, they would be killed by the bacteriophage sprayed on the plate before the delay was over. As the spraying procedure is performed later and later, more and more mutants would have initiated growth, expressed their resistant phenotype, and survived the application of phage. Similar considerations apply to studies of the delayed appearance of streptomycin resistant mutants,[17] streptomycin independent mutants,[18] and prototrophic mutants[19] in *E. coli*.

The delayed initiation of the growth of mutant bacteria does not, however, readily explain the delayed appearance of sectors in colonies. In the experiments of Kaplan[10] on *Serratia marcescens* each colony was presumably derived from a single bacterium; yet bacteria from colonies with induced mutant sectors gave rise to new colonies with an increased incidence of sectoring. In the studies of Witkin[8] and Newcombe,[2] induced lac$^-$ sectors were found in colonies formed mostly from bacteria containing progeny of only one of the nuclei in the original treated bacteria. This was achieved in some experiments by spraying phage $T_1$ on plates containing the treated bacteria some hours after the latter were thought to have begun their divisions. Only those daughter bacteria containing a nucleus conferring phage resistance would be expected to survive, but, nevertheless, lac$^-$ sectors appeared in the resulting colonies with more than spontaneous frequency.

It seems most probable that such sectors are the result of delayed mutation.[2, 8] In at least the last two cases this may be the result of cell rather than gene instability;[20–23] the mutational event may be the delayed consequence of some induced metabolic disorder.

Newcombe[2] has attempted to estimate the magnitude of the contribution to the delayed appearance of phage-resistant mutants made by phenotypic lag, segregation lag, and delayed mutation. It seems unlikely that these factors can account for a delay of more than three to five generations and they could not be responsible, therefore, for the longer delays found by Demerec.[11, 17] Newcombe interprets the discrepancy as being due in part to a selection artifact which under certain conditions causes large numbers of resistant colonies of spontaneous origin to arise where only induced mutants have been expected.[24] At the same time he correctly recognized that the discrepancy might be accounted for by a delay in the onset of division specific for the mutants. This explanation may be especially important in those cases where a selection artifact *may* not operate.[17–19] In any event, the role played by the delayed onset of division of mutants ought to be investigated in each case before other interpretations of the delayed appearance of mutants are adopted.

*Summary.*—Mutants newly arising after heavy ultra-violet irradiation undergo a variable delay in the onset of division that lasts for many generations after their parents have begun to divide. In this respect they also differ from old mutants that had begun their growth before they were irradiated. The length of the delay is too long to be accounted for by the segregation of genetic units, nor is it due to the requirement for developing the mutant phenotype. It is suggested that the mutations occurred among only a fraction of the irradiated bacteria which received an injury that at the same time delayed the onset of growth and made mutation likely. This phenomenon may account for other cases of delayed appearance of mutants in bacterial cultures that have been treated with mutagens.

* This work was supported in part by an American Cancer Society grant recommended by the Committee on Growth of the National Research Council and by a research grant from the National Institute of Health, U. S. Public Health Service.

[1] Atwood, K. C., Schneider, L. K., and Ryan, F. J., these PROCEEDINGS, **37,** 146–155 (1951); Ryan, F. J. (in preparation).

[2] Newcombe, H. B., *Genetics*, **38,** 134–151 (1953).

[3] Lieb, M., *Ibid.*, **36,** 460–477 (1951).

[4] Kaplan, R. W., *Naturwissenschaften*, **23,** 547 (1950).

[5] That the lag periods of the bacteria surviving irradiation were not increased considerably may seem surprising. It must be remembered, however, that in these experiments the surviving bacteria were crowded among multitudes of killed cells which may well have released stimulating substances into the environment. Not only were the lag periods brief, but the generation times were shorter (*ca.* 55 minutes) than those observed for unirradiated unconcentrated bacteria.

[6] Newcombe, H. B., *Genetics*, **33,** 447–476 (1948).

[7] Tatum, E. L., *Cold Spring Harbor Symp. Quant. Biol.*, **11,** 278–284 (1946).

[8] Witkin, E. M., *Ibid.*, **16,** 357–372 (1951).

[9] Newcombe, H. B., and Hawirko, R., *J. Bact.*, **57,** 565–572 (1949).

[10] Bryson, V., and Davidson, H., these PROCEEDINGS, **37,** 784–791 (1951).

[11] Demerec, M., and Latarjet, R., *Cold Spring Harbor Symp. Quant. Biol.*, **11,** 38–50 (1946).

[12] Lederberg, J., these PROCEEDINGS, **35,** 178–184 (1949).

[13] Wagner, R. P., and Haddox, C. H., *Am. Nat.*, **85,** 319–330 (1951).

[14] Demerec, M., *et al.*, *Carnegie Inst. Wash. Yearbook*, **51,** 193–205 (1952).

[15] Weidel, W., *Zschr. Naturforschung*, **7b,** 145–148 (1952).

[16] Demerec, M., these PROCEEDINGS, **32,** 36–46 (1946).

[17] Demerec, M., *Genetics*, **36,** 585–597 (1951).

[18] Labrum, E. L., these PROCEEDINGS, **39,** 280–288 (1953).

[19] Demerec, M., and Cahn, E., *J. Bact.*, **65,** 27–36 (1953).

[20] Kaplan, R. W., *Zschr. Naturforschung*, **7b,** 291–304 (1952).

[21] Auerbach, C., *Cold Spring Harbor Symp. Quant. Biol.*, **16,** 199–214 (1951).

[22] McElroy, W. D., and Swanson, C. P., *Quart. Rev. Biol.*, **26,** 348–363 (1951).

[23] Witkin, E. M., these PROCEEDINGS, **39,** 427–433 (1953).

[24] This artifact counters the objections of Demerec and Flint (*Microbial Genetics Bull.*, **4,** 7 (1951), quoted with permission of the authors) against the possible role of irregularities in the onset of division of treated bacteria (mutant and non-mutant) proposed by Newcombe and Scott (*Genetics*, **34,** 475–592 (1949)). These objections were based on the observation that the over-all delay in the appearance of phage-resistant mutants (where the artifact operates) is greater than the delay, in the same culture, for the appearance of streptomycin-resistant mutants (where the artifact does not operate). Witkin[8] has also objected to the role of irregularities in the onset of division on the basis of data interpreted to indicate that the proportion of whole to sectored mutant colonies is a function of the time of application of phage. These data (her table 5) show no difference,[5] however, at the 5 per cent level of significance and, furthermore, Newcombe[2] found no difference in similar experiments.

# *INDEPENDENT X-RAY EFFECTS ON CHROMOSOME BREAKAGE AND REUNION**

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Following the discovery by Thoday and Read[1] that x-ray induced chromosome aberrations are less frequent when irradiation is performed under anoxic conditions, it seemed reasonable at first to attribute the effect to decreased chromosome breakage. This leads to the expectation that the relations between the total frequency of breaks and the various end results of chromosome breakage should remain unchanged. In other words, the effects of a dose of radiation given in the absence of oxygen should be equivalent to those of a smaller dose given under normal oxygen tension, the ratio of the doses being the same for all effects. As a practical matter however, the frequency of chromosome breaks cannot be observed directly, but is inferred from those events other than restitution; that is, those which do not restore the original configuration. The observed result is a function of the frequency of restitution as well as of the frequency of broken ends. The simple dose-reduction hypothesis has been brought into serious doubt because of recent experiments in which different consequences of chromosome breakage have been compared in the same material. For instance, Schwartz[2] has found that the protective effect of a nitrogen atmosphere during x-ray treatment of maize pollen is much greater for the resultant endosperm mosaics than for complete loss of the genetically marked chromosome segment. Baker, *et al.*,[3–5] have compared x-ray-induced translocations, dominant lethals, and recessive lethals in Drosophila, and have found that the protection afforded by a nitrogen atmosphere, expressed as dose reduction, is different in each case. The changes in quantitative relations among the different effects scored are very difficult to explain on the basis that the probability of breakage is the only factor dependent on the oxygen tension. It has, therefore, been suggested that the ability of the breaks to rejoin is affected.[6] Since it has been shown by Giles and Riley[7] that oxygen tension is influential only *during* irradiation, at the time the breaks (or latent breaks) are formed, and not later during the time of rejoining, any difference in the ability of the breaks to rejoin must be assumed to reside in some property of the breaks themselves, or in some persistent condition brought about by the radiation independently of breakage. The findings to be presented here are inconsistent with the notion that qualitative differences between the breaks account for the oxygen effect, but they indicate that there is an independently induced delay in rejoining.

In these experiments the yields of two-hit aberrations, induced in interphase and scored at metaphase of the first root tip mitosis in *Vicia faba*, were studied with different combinations of dose fractionation and treatment with 2,3-dimercaptopropanol (BAL). The effect of BAL on this system may be considered essentially the same as that of anoxia, since it has been shown[6] that both treatments produce quantitatively similar effects on x-ray damage, and that when BAL is combined with in vacuo irradiation there is little or no greater protective effect than with either treatment alone. Aberrations of both the one-hit and the two-hit varieties are

reduced in frequency by BAL treatment. The results of previous dose fractionation and intensity studies[8] led to the hypothesis that the breaks normally remain open for about 2 hours, and that the presence of BAL in the material during irradiation shortens this period to about $^1/_2$ hour. These time relations cannot be properly regarded as difference in rates of rejoining, because there is a period of greater or lesser duration within which there is no rejoining whatever. It is for this reason that fractionation of the dose provides a simpler experimental approach than does variation of the intensity. It will be shown here that an initial irradiation without BAL delays the rejoining of breaks produced by further irradiation in the presence of BAL. This suggests a mutual independence of initial breakage and reunion delay.

TABLE 1

RESULTS OF DOSAGE FRACTIONATION STUDIES

Dose in r units. 300 cells scored for each treatment.

| EXPT. NO. | FIRST FRACTION OF X-RAYS GIVEN AT TIME $t$ | SECOND FRACTION OF X-RAYS GIVEN AT $t$ + 30 MIN. | THIRD FRACTION OF X-RAYS GIVEN AT $t$ + 60 MIN. | OBSERVED[a] ABERRATION FREQ./CELL | EXPECTED FREQ./CELL IF ALL BREAKS INTERACT (EQ. 1) | EXPECTED FREQ./CELL IF ALL FRACTIONS ARE ADDITIVE |
|---|---|---|---|---|---|---|
| 1 | Alone, 400 | ... | ... | 0.087 | 0.078 | ... |
| 2 | With BAL, 400 | ... | ... | 0.043 | 0.041 | ... |
| 3 | Alone, 800 | ... | ... | 0.333 | 0.335 | ... |
| 4 | With BAL, 1000 | ... | ... | 0.263 | 0.258 | ... |
| 5 | Alone, 400 | With BAL, 400 | ... | 0.263 | 0.233 | 0.119 |
| 6 | Alone, 400 | With BAL, 200 | With BAL, 200 | 0.270 | 0.233 | 0.098 |
| 7 | With BAL, 400 | With BAL, 400 | ... | 0.153 | 0.165 | 0.082 |
| 8 | With BAL, 400 | With BAL, 200 | With BAL, 200 | 0.090 | 0.165 | 0.061 |
| 9 | With BAL, 500 | With BAL, 400 | ... | 0.213 | 0.209 | 0.105 |
| 10 | With BAL, 500 | With BAL, 200 | With BAL, 200 | 0.147 | 0.209 | 0.084 |
| 11 | Alone, 400 | ... | Alone, 400 | 0.353 | 0.335 | 0.156 |
| 12 | Alone, 400 | ... | With BAL, 400 | 0.233 | 0.233 | 0.119 |
| 13 | With BAL, 600 | ... | With BAL, 400 | 0.133 | 0.258 | 0.134 |
| 14 | With BAL, 400 | With BAL, 400 | With BAL, 200 | 0.293 | 0.258 | 0.091 |
| 15 | With BAL, 600 | ... | ... | 0.093 | 0.092 | ... |

[a] For all cases in which there was interaction, the observed value fell within the 95 per cent confidence limits of the theoretically expected value.

*Materials and Methods.*—Seeds of *Vicia faba* were soaked in water for 24 hours previous to irradiation. BAL treatment consisted in soaking for 30 minutes in a $2 \times 10^{-3}$ $M$ solution of BAL immediately prior to irradiation except in experiments 16, 17, and 18 where presoaking was reduced to 15 minutes. Three days after irradiation, at the time of the first root tip divisions, the tips were cut off and placed for 6 hours in a 0.2 per cent colchicine solution, then fixed overnight in acetic alcohol. Acetocarmine smears were made and 300 metaphases were scored for each datum. The aberrations scored were rings and dicentrics. A more detailed description of techniques has been given elsewhere.[6]

All irradiations were administered with a G. E. Maxitron tube operated at 250 kvp with 3mm. of Al filtration. The dose rate for all irradiations in tables 1 and 2 was 200 r per minute, and the intervals between fractions were $^1/_2$ hour each.

*Results and Discussion.*—The results of the fractionation experiments are given

in tables 1 and 2 where the protocols of each experiment are summarized and the data are expressed as aberration frequencies per cell.

Each chromosomal aberration scored in the present study is believed to be a result of the interaction of two breaks. This is reflected not only in that the rearrangements involve two separate points of breakage, but also in that the square root of the frequency of the aberrations is, under constant conditions of irradiation, directly proportional to dose.[8] As a hypothetical model serving to illustrate the rationale of this dose-squared relation, consider a small volume within the nucleus and assume that if two breaks lie within this volume they will rejoin to form a rearrangement. If there is a probability, $p$, that a break lies within this volume, then there will be a probability $p^2$ that two breaks simultaneously lie within the volume, e.g., that a rearrangement is formed. It is assumed that $p$ is proportional to the number of breaks, and thus to the dose. This proportionality might be different under different conditions of irradiation, however, since in actuality $p$ may relate to many things other than spatial location. Where it is proper to assign probabilities of interaction, the total yield, $Y$, to be expected from combining $n$ treatments which produce two-hit aberration frequencies $x_1$, $x_2$, ..., $x_n$ respectively is given by

$$Y = (x_1^{1/2} + x_2^{1/2} + \ldots + x_n^{1/2})^2 \qquad (1)$$

provided that the effects of the $n$ treatments are coincident in time. However, if the dose is delivered at sufficiently low intensity so that some of the breaks rejoin before the conclusion of the irradiation the aberration frequency will no longer be proportional to the square of the dose, but will fall more or less below the expected values, a phenomenon discussed extensively by Sax and Brumfield.[9] In the limiting case, such as the division of the dose into two fractions separated by a time interval sufficient to allow complete reunion of the first set of breaks, the yield of the fractions will be simply additive, since there is no interaction between them. Fractionation effect is conveniently defined according to the preceding statements. If the yield of the combined treatments conforms to equation (1) there is said to be no fractionation effect, but if the yield is additive, there is said to be a complete fractionation effect.

It is apparent from table 1 (experiments 5, 6, 11, and 12) that where the first fraction was given without BAL, there was no fractionation effect, e.g., there was complete interaction between the breaks produced by the first and by the subsequent fractions given in the presence of BAL as much as 1 hour later. This means that breaks produced in the BAL-treated material which can interact with each other can also rejoin just as efficiently with other breaks produced in the absence of BAL. Where the first fraction was given in the presence of BAL, however, there was marked failure of interaction in experiments carried beyond 30 minutes; that is, when a portion of the dose was given as a third fraction as in experiments 8, 10, and 13. In these experiments the time interval of 1 hour between the first and third fractions is sufficient to exclude breaks produced by the third fraction from participating in rearrangements with those of the first and possibly the second fractions. The data from experiments 8 and 10 fit best the hypothesis that the first two fractions interact and the third is additive. Since the first and second fractions do interact, the question arises of why the second fraction does not further extend

the delay so that the breaks of all three fractions would be open simultaneously. In the experiments summarized in table 2, the time dependences of the effects at two different doses are compared. When a dose of 600 r is given over a period of 3 hours, the frequency of aberrations is below that obtained with 600 r at high intensity, whereas the aberration yield of 900 r is the same whether the dose is delivered in 5 minutes or in 3 hours. This suggests that the delay in rejoining is itself dose dependent. Therefore, it might be expected that the delay of about 30 minutes produced by 400 r with BAL would be extended for less than 30 minutes by a second fraction of 200 r as in experiments 8 and 10, table 1. This interpretation is supported by experiment 14; when the second fraction was increased to 400 r the fractionation effect disappeared. In this experiment the second fraction maintained the broken ends produced by the first for a time sufficient to insure their interaction with the third fraction.

The question arises of whether the frequency of rearrangements is influenced by the time required for rejoining, since broken ends open for a long time might move apart and therefore become more likely to participate in a rearrangement and less

TABLE 2

NUMBER OF ABERRATIONS PER CELL PRODUCED AS INTENSITY OF RADIATION DECREASES

| DOSE (r) GIVEN IN $t$ MINUTES | $t$ | NO. ABERRATIONS/ CELL |
|---|---|---|
| 600 | 3 | 0.177 |
| | 60 | 0.193 |
| | 120 | 0.157 |
| | 180 | 0.093 |
| 900 | 5 | 0.420 |
| | 60 | 0.436 |
| | 180 | 0.433 |

TABLE 3

THE EFFECT OF VARYING THE TIME BETWEEN FRACTIONS

| EXPT. NO. | TIME BETWEEN FIRST DOSE (400 r) AND SECOND DOSE (400 r WITH BAL), MIN. | ABERRATIONS FREQUENCY PER CELL[b] |
|---|---|---|
| 16 | 15 | 0.240 |
| 5[a] | 30 | 0.263 |
| 12[a] | 60 | 0.233 |
| 17 | 60 | 0.253 |
| 18 | 90 | 0.273 |

[a] From table 1.

[b] There is no significant difference between any of the figures in this column.

likely to restitute the original configuration. It is therefore of interest to see to what extent the reduction in reunion delay accounts for the reduction in aberration frequency in BAL treated material. If it fully accounts for the difference, then an orderly relation among delay time, initial break frequency, and aberration yield is implied. In experiments 12 and 13 in table 1 the doses in the first fractions with and without BAL were adjusted to give about the same aberration frequencies, 8.7 and 9.7 per cent respectively. The next fraction, given after 1 hour, was 400 r with BAL in both cases. In experiment 12 there is no fractionation effect, but in experiment 13 the effect is complete. Thus the delay is greater than 1 hour in one case and less than 1 hour in the other, although the aberration frequencies are essentially the same. It could be argued, *ad hoc*, that the number of broken ends available as a result of the first irradiation in experiment 13 is greater than in experiment 12 by an amount that is exactly compensated by the decrease in delay time. Since it has been shown that the delay time is itself a function of dose, the quantitative formulation of such an hypothesis would be rather involved. The result of further experiments to test the notion are given in table 3, together with pertinent experiments from table 1. In each case an initial dose of 400 r was given,

producing a fixed number of broken ends and a relatively long delay time. After various time lapses ranging from 15 minutes to $1^1/_2$ hours during which only these first breaks were open, a second group of broken ends was introduced by means of 400 r with BAL, to remain open concurrently with the first until the time of reunion. This second dose produces, by itself, a relatively short delay time. Whether the delay caused by the second fraction is assumed to run concurrently with the first, or consecutively, it is clear that the relation between the number and the duration of broken ends is not the same in the different experiments. The aberration yields, however, are unchanged. The hypothesis that the aberration yield depends on a systematic relation between break frequency and delay time is therefore untenable, if, as we assume, the broken ends are actually open during the time between irradiation and rejoining. In the present experiments all that can be seen is the absence of fractionation effect. This could be due either to a delay in the opening of latent breaks or to a delay in rejoining of breaks formed during the course of the radiation. We prefer the latter interpretation. In other material the delay time may have an important influence on the frequency of rearrangements, but in the experiments reported it does little or nothing else than determine the response of the system to dose fractionation. We conclude, for the present, that in this system the difference in aberration yield caused by BAL reflects a difference in initial break frequency.

Aberration production and reunion delay are both ameliorated by BAL, but to different degrees. It is clear from experiments 12 and 13 that the effect of BAL is greater, in terms of dose reduction, for delay than for aberration frequency; when the doses with and without BAL are adjusted to equal aberration yields, BAL still shortens the delay time. It has also been shown that breaks induced in the presence and in the absence of BAL interact with each other to give precisely the expected aberration yields, so that an explanation based on qualitative differences between the broken ends presents some difficulty. In short, the x-ray effects on breakage and on reunion appear to be quite independent of each other. The question arises, then, of the mechanism through which x-rays can produce a delay in the reunion of chromosome breaks. If reunion required nothing more than the chance association of broken ends, the observed time relations would be quite anomalous. The fact that the delay is dose dependent and that it responds to adjunctive treatment suggests that reunion is not a passive process, but requires some metabolic activity which can be impaired by radiation. The specific system would have to be one which is normally in operation, not called forth by chromosome breakage. In comparison with most aspects of cellular metabolism, the hypothetical process affected would have to be unusually radiosensitive. The synthesis of desoxyribonucleic acid (DNA) has been shown to be rather easily impaired by radiation,[10] and the obvious relation of DNA synthesis to the chromosomes lends some interest to the notion that the effect on reunion delay may concern this process. Finally, it is to be wondered whether reunion delay is another manifestation of the same type of radiation injury which causes mitotic delay[11] where a relatively sensitive process is inhibited, but in which the cells eventually recover.

*Summary.*—Some effects of x-ray dose fractionation on two-hit chromosome aberrations in root tips of *Vicia faba* have been studied with and without the protective agent, BAL. The data are consistent with the following conclusions.

1. The time interval between breakage and reunion is dose dependent and is decreased if BAL is present during irradiation.

2. Breaks produced in the presence of BAL interact normally with those produced in its absence.

3. Rejoining of breaks produced in the presence of BAL is delayed by previous irradiation in its absence.

4. BAL decreases the aberration yield and the reunion delay by different amounts.

5. The reunion delay time *per se* has little or no influence on the aberration frequency.

Taken together, the findings indicate that chromosome breakage and the radiation-induced delay of reunion are different and independent effects of the radiation.

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[1] Thoday, J. M., and Read, J., *Nature*, **160,** 608 (1947).
[2] Schwartz, D., these PROCEEDINGS, **38,** 490–494 (1952).
[3] Baker, W. K., and Sgourakis, E., *Ibid.*, **36,** 176–184 (1950).
[4] Baker, W. K., and Von Halle, E. S., *Ibid.*, **39,** 152–161 (1953).
[5] Baker, W. K., and Edington, C. W., *Genetics*, **37,** 665–667 (1952).
[6] Wolff, S., *Genetics* (in press).
[7] Giles, N. H., Jr., and Riley, H. P., these PROCEEDINGS, **36,** 337–344 (1950).
[8] Wolff, S., *Nature*, **173,** 501–502 (1954).
[9] Sax, K., and Brumfield, R. T., *Am. J. Botany*, **30,** 564–570 (1943).
[10] Hevesy, G., *Symposium on Radiobiology*, John Wiley & Sons, New York, 1952, pp. 189–213.
[11] Gaulden, M. E., Nix, M., and Moshman, J., *J. Cellular Comp. Physiol.*, **41,** 451–470 (1953).

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## *THE SEPARATION AND ISOLATION OF PARTICULAR BIOCHEMICAL MUTANTS OF NEUROSPORA BY DIFFERENTIAL GERMINATION OF CONIDIA, FOLLOWED BY FILTRATION AND SELECTIVE PLATING**

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The importance of biochemical mutant strains of micro-organisms for purposes of bioassay and for fundamental investigations of gene action has motivated many efforts to devise means for procuring such strains with ease and efficiency. The general problem is to separate mutant from non-mutant nuclei out of a population that has been treated with a mutagen. In Neurospora there have been two principal methods for isolating biochemical mutants, the original procedure of Beadle and Tatum,[1] and a method developed somewhat later by Lein, Mitchell, and Houlahan.[4] The former is laborious and not readily adapted to selecting particular mutant types. The latter permits a preliminary screening of wild-type from mutant nuclei; but like the former it involves a sexual cross as part of the procedure, with resultant loss in time and a possible loss of mutants that do not readily pass through the sexual stage. The object of the present investigation has been to develop a method which would allow the direct isolation of large numbers of biochemical mu-

tant strains in Neurospora with procedural efficiency, and with possibilities for selection of particular types. It was believed that a method having these attributes might permit the isolation of a wider array of biochemical mutants. The primary objective in many respects has been realized with the development of a filtration and selective plating technique. A search for new mutant types has not yet been pressed, but certain preliminary efforts warrant optimism.

The idea of placing irradiated conidia of a fungus into a minimal medium and filtering off mycelium as the wild type spores germinate was utilized first with *Ophiostoma multiannulatum* by Fries.[3] Fries proposed that this principle could be adapted to any fungus possessing filamentous mycelia. Initial attempts to apply the principle to Neurospora were unsuccessful,[4] apparently because the mutant conidia failed to maintain viability for sufficient periods of time, and because mutants were lost through heterocaryosis. These difficulties have for many practical purposes been overcome in the present work. Preliminary results were reported earlier.[7]

## Materials and Methods

*Strains.*—Various strains of Neurospora were utilized in experiments designed to recover induced biochemical mutants. It was recognized at the outset that isolations of mutagenically treated multinucleate conidia would be likely to yield heterocaryons of wild-type and mutant nuclei rather than homocaryotic mutant cultures. Since the procedure utilized for recovery of mutants would seem to entail the filtering away of such heterocaryons, early experiments were performed with a microconidial (mononucleate) strain obtained from N. Giles of Yale University. Under the conditions of the experiments, this strain proved to be unsuitable because of poor viability of the conidia after several days retention in liquid media. Subsequent work, therefore, was done entirely with macroconidial strains. Culture W-1, obtained as a wild-type segregant from a cross made in this laboratory, was found to produce a preponderance of mono- and dinucleate conidia, and was used with success until replaced by the more vigorous wild-type strain 74A-3b. The latter strain, isolated by P. St. Lawrence of Yale University, also produces a high frequency of mono- and dinucleate conidia. Presumably, most of the recovered mutants had their origin in such conidia rather than in conidia having higher nuclear numbers. All of the reported data referring to the recovery of induced mutants were obtained with one or the other of these wild-type strains.

The mutant strains utilized in experiments to test recovery of mutants from synthetic mixtures of conidia of different types were: 7-5, which grows on cirtulline or arginine; 14-1, which grows on ornithine, citrulline, or arginine; purple adenineless; 1-13 (adenineless); and 5531A (pantothenicless). All of the strains except 5531A were isolated in this Laboratory. Strain 5531A was received from F. J. Ryan of Columbia University.

*Media.*—Unless otherwise started, the media utilized in this study were those described by Beadle and Tatum.[5] In certain instances of the experimentation, the standard minimal medium was supplemented with 2.01 g. monobasic potassium phosphate and 0.08 g. dibasic sodium phosphate to each liter of Fries' solution to stabilize the pH at 5.5. Coconut milk factors for supplementing media were provided by F. C. Steward of Cornell University.

*Procedure for Recovery of Induced Mutants.—Basic Principles:* (1) Biochemical

mutant and wild-type conidia can be separated from a mixture of the two by placing the mixture into minimal medium and filtering off the wild-types as they germinate; (2) the mutant spores can be isolated and preserved by plating onto supplemented media designed to permit growth of particular mutant types.

*Specifically:* Conidia were taken from cultures that had grown on 20 ml. of minimal agar in 125-ml. Erlenmeyer flasks for a period of five days at 25°C. Between 50 and 100 million of the conidia were suspended in 30 ml. of sterile distilled water and placed in a Petri plate for exposure to ultra-voilet light. Irradiations were made with a 15-watt G-E ultra-violet germicidal lamp housed in a sterile chamber. Measurements with an intensity meter indicated that for different experiments dosages ranged from 70 to 100 milliwatts per square foot. Times of treatment ranged from 30 to 90 minutes. No treatment optima have yet been determined. During the irradiation period, the spore suspension was agitated by means of an electric vibrator attached to the stand supporting the suspension. After irradiation, the conidial suspension was inoculated into a 1-liter Erlenmeyer flask containing 500 ml. of phosphate-buffered minimal medium. The flask was incubated in a sterile chamber, equipped with built-in rubber gloves to facilitate aseptic manipulations. Aeration of the inoculated medium was provided by a stream of air passed through a sterile glass-wool filter. At the beginning of each experiment the chamber was supplied with a number of sterile 1-liter flasks equipped with filters. The filters consisted of two layers of cheesecloth secured to the mouth of each flask with a rubber band and forced inward until they assumed a cup shape. Whenever mycelia were appreciably perceptible to the naked eye, the contents of the incubation flask were poured into one of the empty flasks through the cheesecloth filter, and the filtrate was again incubated under aeration. Usually no filtrations were made during the first 18 hours of incubation; during the second 18-hour period filtrations were made every 3 to 6 hours; subsequently, filtrations were made at 6- to 12-hour intervals. After filtering off most of the conidia that would germinate in the incubation flask, the remaining conidia were plated onto minimal agar medium containing supplements that permitted growth of the desired mutant types. This step is similar to the procedure for plating microconidia described by Tatum, Barratt, Fries, and Bonner.[6] All plating media included 1 per cent sorbose, which induced colonialization of the growth centers, *see Biblio.* Tatum, Barratt, and Cutter.[5] After the plates had been incubated for approximately 24 hours at 25°C., isolations from individual colonies were made into culture tubes containing appropriately supplemented media. Identification of the culture isolates followed the principles outlined by Beadle and Tatum.[1]

*Procedure for the Recovery of Mutants from Synthetic Mixtures.*—To explore the efficiency of recovery of mutants by the filtration and selective plating method, the procedures of the method were applied to synthetic mixtures of wild-type and known mutant conidia not treated with a mutagen. Estimates of the number of conidia of the different genotypes were based on hemocytometer counts; and by appropriate dilution, known mixtures of mutant and wild-type conidia were obtained. In these experiments, 50 million spores was the maximum inoculum placed in the aeration flask. At the beginning of the incubation period, and every 24 hours thereafter, aliquots of the incubation medium were plated to permit determination of change in proportions of viable wild-type and mutant conidia.

## RESULTS

The results are presented in two sections. The first section shows the results of experiments designed to recover biochemical mutants after treatment of the wild-type conidia with a mutagen. The second section illustrates certain facets of the filtration method, revealed by the recovery of mutants from synthetic mixtures of mutant and wild-type conidia.

TABLE 1

KINDS OF MUTANT STRAINS ISOLATED AND RELATIVE EFFECTIVENESS OF RECOVERY AFTER TREATMENT WITH ULTRA-VIOLET LIGHT

| METABOLITE REQUIRED BY THE MUTANT | NUMBER OF ISOLATES MADE | NUMBER OF SPECIFIC MUTANTS RECOVERED | $M/T$[a] |
|---|---|---|---|
| Arginine | 257 | 53 | 0.206 |
| Methionine | 464 | 39 | 0.084 |
| Lysine | 306 | 31 | 0.101 |
| Leucine | 160 | 7 | 0.044 |
| Proline | 16 | 2 | 0.125 |
| Tryptophane | 20 | 1 | 0.040 |
| Pantothenic acid | 70 | 6 | 0.086 |
| Thiamine | 84 | 6 | 0.071 |
| Riboflavin | 78 | 4 | 0.051 |
| Inositol | 136 | 9 | 0.066 |
| Nicotinic acid | 275 | 3 | 0.011 |
| Choline | 48 | 1 | 0.020 |
| *p*-Amino benzoic acid | 55 | 1 | 0.018 |
| Adenine | 76 | 14 | 0.183 |
| Guanine | 84 | 4 | 0.047 |

[a] Number of specific mutants recovered/number of isolates made.

TABLE 2

MUTANTS RECOVERED FROM COMPLETE AGAR PLATES FOLLOWING TREATMENT WITH ULTRA-VIOLET LIGHT

| | NUMBER OF MUTANTS IN EACH GROUP | NUMBER OF MUTANTS/ NUMBER CLASSIFIED |
|---|---|---|
| Classified: | | |
| Grow on casein | 3 | 0.090 |
| Grow on vitamins | 1 | 0.030 |
| Grow on sodium nucleate | 8 | 0.235 |
| Grow on complete only | 22 | 0.645 |
| Unclassified | 50 | ... |

*Recovery of Induced Mutants.*—Various mutant strains that have been isolated by means of the filtration method after treatment of conidia with ultra-violet light are listed in table 1. The table summarizes results of a number of experiments. The different individual experiments involved different dosages of mutagen, either wild-type strain W-1 or 74A-3b, variable sizes of inoculum, different periods of incubation, and different intervals of filtration. The compositing of data seems justified since no one set of the conditions used represents maximum effectiveness of the method. Thus, the summary only represents the array of mutant types recovered over the experimental series. In the experiments summarized, each plating was made on minimal agar supplemented with a specific

nutrient. Selection of the corresponding mutant types was high, but in numbers of instances mutants requiring nutritional supplements other than those supplied in the plating medium were recovered. For example, a leucineless mutant was found on a plate specifically supplemented with arginine. The fraction $M/T$ (number of viable specific mutants/total isolates) is expressed as a frequency. Argineless mutants were recovered most efficiently, and nicotinicless mutants proved to be the most difficult to recover of types that were persistently sought. A number of the mutants listed in table 1, including guanineless and nicotinicless, have been crossed to wild-type, and approximately one-half of the progeny showed the particular biochemical deficiency.

TABLE 3

KINDS OF MUTANT STRAINS ISOLATED AND RELATIVE EFFECTIVENESS OF RECOVERY AFTER TREATMENT WITH APPROXIMATELY 8000 r UNITS OF X-RAY

| METABOLITE REQUIRED BY THE MUTANT | NUMBER OF ISOLATES MADE | NUMBER OF SPECIFIC MUTANTS RECOVERED | $M/T$ |
|---|---|---|---|
| Inositol | 50 | 1 | 0.020 |
| Methionine | 45 | 5 | 0.111 |
| Lysine | 56 | 7 | 0.125 |
| Nicotinic acid | 86 | 1 | 0.011 |

TABLE 4

USE OF THE FILTRATION TECHNIQUE WITH SYNTHETIC MIXTURES TO INCREASE PROPORTIONS OF MUTANT TO WILD-TYPE CONIDIA

Pantothenicless and lysineless conidia were mixed with wild-type so that each of the former constituted approximately 1 per cent of the total number of conidia incubated. Alterations in proportions were detected by isolations from two series of supplemented minimal plates, one with lysine and one with pantothenate.

| | RECOVERY FROM PANTOTHENATE PLATES | | RECOVERY FROM LYSINE PLATES | | $M/T$ | |
|---|---|---|---|---|---|---|
| DAYS | NUMBER OF ISOLATES MADE | NUMBER OF SPECIFIC MUTANTS RECOVERED | NUMBER OF ISOLATES MADE | NUMBER OF SPECIFIC MUTANTS RECOVERED | PANTOTHENATE PLATES | LYSINE PLATES |
| 0 | 56 | 0 | 56 | 0 | 0.00 | 0.00 |
| 1 | 56 | 1 | 56 | 2 | 0.02 | 0.04 |
| 2 | 56 | 16 | 56 | 18 | 0.29 | 0.32 |
| 3 | 56 | 41 | 56 | 46 | 0.73 | 0.80 |
| 4 | 56 | 51 | 56 | 53 | 0.91 | 0.95 |
| 5 | 56 | 56 | 56 | 56 | 1.00 | 1.00 |

In some experiments platings were made onto complete agar. Most of the mutants obtained were identified only as to the general category of growth requirement that resulted from mutation. A high proportion of the mutants failed to give growth tests on any of the discriminatory media employed. It would appear that many mutants having complex or unusual growth factor requirements may be recovered by plating onto complete media. The data are summarized in table 2.

To determine whether the filtration method is effective for recovering mutants after treatment with a standard mutagen other than ultraviolet light, an experiment utilizing x-ray treatment of wild-type conidia was performed. The results are found in table 3. The effectiveness of recovery of a sample of mutant types compares favorably with the effectiveness obtained with ultra-violet light as a mutagen.

*Recovery of Mutants from Synthetic Mixtures.*—The data presented in table 4 were obtained from experiments designed to recover lysineless and pantothenicless conidia from a synthetic mixture with wild-type. Initially, the different mutant conidia were incorporated with wild-type so that each of the former constituted approximately 1 per cent of the total number of conidia incubated. This approximation designates the total number of conidia placed into the incubation flask: it does not represent the total number of viable spores incubated. The effectiveness of recovery of the specific mutant types is shown by the daily increase in the numbers of mutants isolated. Nothing is shown, however, concerning the daily absolute population changes of either the mutant conidia or of the wild-type conidia.

TABLE 5

RECOVERY OF MUTANTS FROM SYNTHETIC MIXTURES

Mixtures of different mutant conidia were made with conidia of wild-type strain 74A-3b, and incubated in minimal medium. The effects of the filtration technique on proportion of types in the spore population were estimated on the basis of daily samples plated on appropriate media.

| | | ACTUAL ISOLATIONS | | CALCULATED CONIDIAL POPULATIONS[a] | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | TOTAL CONIDIA[b] | WILD-TYPE CONIDIA[c] | | MUTANT CONIDIA[d] | | $M/T$[e] | |
| MUTANT STRAIN | DAYS | TOTAL | NO. OF MUTANTS | (NO VIABLE × $10^{-3}$) | (NO. VIABLE × $10^{-3}$) | % OF ORIGINAL | (NO. VIABLE × $10^{-3}$) | % OF ORIGINAL | ACTUAL ISOLATIONS | CALCULATED |
| purple ad-less | 0 | 80 | 0 | 56,000. | 55,000. | 100. | 1,000. | 100. | 0.00 | 0.02 |
| | 1 | 76 | 1 | 1,600. | 1,050. | 1.9 | 550. | 55. | 0.13 | 0.34 |
| | 2 | 80 | 22 | 9. | 1.5 | 0.003 | 7.5 | 0.75 | 0.28 | 0.83 |
| | 3 | 47 | 30 | 9. | 1.5 | 0.003 | 7.5 | 0.75 | 0.64 | 0.83 |
| | 4 | 30 | 19 | 5.2 | 0.5 | 0.001 | 4.7 | 0.47 | 0.63 | 0.90 |
| | 5 | 14 | 9 | ... | ... | ... | ... | ... | 0.64 | ... |
| 1-13 | 0 | 80 | 0 | 57,500. | 49,500. | 100. | 8,000. | 100. | 0.00 | 0.14 |
| | 1 | 80 | 1 | ... | ... | ... | ... | ... | 0.01 | ... |
| | 2 | 80 | 3 | 610. | 390. | 0.79 | 220. | 2.8 | 0.04 | 0.36 |
| | 3 | 80 | 11 | 690. | 370. | 0.75 | 320. | 4.0 | 0.14 | 0.48 |
| | 4 | 70 | 27 | 45. | 9. | 0.02 | 36. | 0.45 | 0.34 | 0.80 |
| 14-1 | 0 | 63 | 1 | 22,000. | 20,000. | 100. | 2,000. | 100. | 0.02 | 0.09 |
| | 1 | 63 | 18 | 100. | 70. | 0.35 | 30. | 1.5 | 0.29 | 0.30 |
| | 2 | 63 | 52 | 70. | 19. | 0.10 | 51. | 2.6 | 0.83 | 0.73 |
| | 3 | 63 | 59 | 71. | 6. | 0.03 | 65. | 3.3 | 0.94 | 0.92 |
| | 4 | 63 | 60 | 73. | 2. | 0.01 | 71. | 3.6 | 0.95 | 0.97 |
| 7-5 | 0 | 80 | 7 | 48,800. | 48,000. | 100. | 800. | 100. | 0.09 | 0.02 |
| | 1 | 80 | 45 | 1,000. | 240. | 0.50 | 760. | 95. | 0.56 | 0.76 |
| | 2 | 80 | 67 | 282. | 22. | 0.05 | 260. | 33. | 0.84 | 0.92 |
| | 3 | 80 | 74 | 255. | 6.6 | 0.01 | 248. | 31. | 0.93 | 0.97 |
| | 4 | 80 | 77 | 224. | 2.4 | 0.005 | 222. | 28. | 0.96 | 0.99 |
| | 5 | 80 | 77 | 204. | 0.8 | 0.002 | 203. | 25. | 0.96 | 0.99 |
| 5571 A | 0 | 80 | 0 | 44,800. | 44,000. | 100. | 800. | 100. | 0.00 | 0.02 |
| | 1 | 80 | 4 | 7,300. | 6,300. | 14. | 1,000. | 100 plus | 0.05 | 0.14 |
| | 2 | 34 | 5 | .660. | 100. | 0.23 | 560. | 70. | 0.15 | 0.85 |
| | 3 | 35 | 16 | 4.2 | 0.2 | 0.000 | 4. | 0.5 | 0.46 | 0.95 |
| | 4 | 37 | 31 | 4.0 | 0.0 | 0.000 | 4. | 0.5 | 0.84 | 1.00 |

[a] Based on plate counts of suitably diluted samples of conidia.

[b] Ten plates containing complete medium were each inoculated with 0.5 ml. of a spore suspension—after incubation at 25°C. plates contained 5–100 colonies.

[c] Ten plates containing minimal medium were each inoculated with 0.5 ml. of a spore suspension—after incubation at 25°C. plates contained 0–100 colonies.

[d] Based on difference between total and wild-type spore counts.

[e] Number of viable mutants/total viable spores.

From the data one may conclude that the increase in proportion of mutant to wild-type conidia results from the elimination of the wild-types by filtration. An experiment similar to that reported in table 4 was made with a nicotinicless mutant strain. At the end of 4 days of incubation the $M/T$ value was 0.467. The low frequency of recovery of nicotinicless mutants may be pertinent to the inefficiency of recovery of induced nicotinicless mutants as reported in table 1.

A more revealing type of experiment is illustrated in table 5, which shows by daily absolute population changes that mutant conidia as well as wild-type conidia are greatly reduced in numbers, but at a much slower rate. During the time of incubation, the wild-type conidia were reduced in numbers to less than 0.02 per cent of the original total in every experiment, while the number of mutant conidia remained above 0.5 per cent of the original total. Although the majority of mutant conidia are lost, the proportion of mutant conidia is greatly increased, a fact that, no doubt, explains the effectiveness of recovery of particular mutants from synthetic mixtures.

## DISCUSSION

Many nutritional mutant types of Neurospora that have been isolated by other methods may be readily recovered by means of the techniques described above. The present method has the following apparent advantages: the possibility of screening vast populations of mutagenically treated nuclei; the elimination of a sexual cross in the isolation of the mutants; ready adaptability of selection of particular desired mutant types; and economy of means in time and equipment. A method having these qualities would seem to offer opportunities for elaborating the spectrum of possible mutant types. For example, mutants responding to coconut milk factors have been recovered by this means.

It is clear, however, that the present method does not permit the recovery of all mutant types with equal efficiency, and may not permit the recovery of certain types at all. This is indicated by the experiments with the synthetic mixtures where conidia of the mutant types were lost rapidly during the time of incubation and filtration (table 5). It is not known whether the mutant conidia from the irradiated populations are lost in the same proportion as they are in the synthetically mixed populations. In fact it seems doubtful that they are, since adenineless mutants are recovered with relative ease from irradiated populations (table 1) but are eliminated rapidly in synthetic mixtures (table 5). The resolution of this seeming anomaly probably lies in the different sets of conditions under which the two types of experiments are run. The fate of mutant conidia that are lost may possibly be accounted for by one, or several, of the following:

*1. Mechanical Elimination of Spores During the Filtration Process:* When allowed to collect in appreciable quantities, the mycelia growing in the incubation flask cause mechanical difficulties during filtration by forming relatively impervious pads on the cheesecloth filters. Such pads conceivably prevent large numbers of conidia from passing through the filter. Analyses of the spore content of these mycelial pads have not been made, but microscopic observations have revealed the presence of conidia adhering to the hyphae.

*2. Formation of Heterocaryons Giving Rise to Mycelia Nutritionally Equivalent to Wild-Type:* Heterocaryosis may deplete the mutant population at any of several

stages during the experimental period. Isolates initially showing a negative growth response in minimal medium, and later developing apparently normally, were crossed to wild-type. Single ascospore isolates from such crosses have shown that mutant types have fused both with wild-types and with other mutant types. It is assumed from such data that many mutants have been lost through the formation of heterocaryons, but the extent of this loss is obscure. Heterocaryosis may be minimized by employing strains which do not form heterocaryons readily.

*3. Failure of Mutant Spores to Survive in Liquid Medium for Extended Periods of Time:* Inviability due to processes resulting from prolonged suspension in a liquid environment may account for the loss of many conidia during incubation. According to data presented by Bowne,[2] the number of mutant conidia surviving incubation at 3 to 5°C. in starvation medium for a three-day period is relatively few. Similar conclusions can be drawn from the data in table 5.

*4. Cross-Feeding of Metabolites:* Products arising from metabolizing and/or lysed spores may supply necessary growth supplements which contribute to the germination of many of the mutant types. If operative, this phenomenon of cross-feeding is no doubt more effective in the elimination of mutant types requiring specific metabolites in relatively minute amounts, e.g., various vitamin requirers. The existence of the phenomenon could be tested by incubating known mutant conidia in media which previously had a population of spores growing in it. Rapid growth in such media would favor the theory of cross-feeding. If it occurs, it may be necessary to centrifuge and wash the spores periodically during incubation, adding new media after each centrifugation.

In addition to the loss of mutant conidia during incubation other factors related to the method remain to be evaluated. For example, the function and utility of aeration have not been established with certainty. Aeration was initially adopted (1) to provide oxygen for rapid germination of the wild-type spores, and (2) to agitate the medium thus reducing the possibilities of heterocaryosis by preventing prolonged contact of conidia. Experiments which have been conducted with the object of comparing the efficacy of the filtration method with and without aeration have favored aeration, and until further data are available it seems justifiable to continue aeration as a part of the procedure.

## SUMMARY

Rapid and efficient recovery of biochemical mutants of Neurospora can be achieved by a method whose principles are essentially those utilized by Fries for the isolation of biochemical mutants of Ophiostoma. The procedure involves: (1) placing a population of irradiated conidia into a liquid medium which permits growth of wild-type conidia but is unfavorable to the germination of desired mutant types; (2) elimination of the germinated spores by passing the conidial suspension through a cheesecloth filter at appropriate intervals; (3) plating the concentrate of mutant spores, after the period of incubation and filtration, onto agar supplemented with metabolites that permit growth of the desired mutant types; and (4) isolation of cultures from colonies on the plate, followed by testing for differences from the nutritional requirements of wild-type. A large variety of different biochemical mutants has been recovered, many with apparently high frequency.

The efficacy of the filtration method as a means of concentrating mutant spores has been explored by applying its techniques to synthetic mixtures of wild-type and known mutant conidia. The results show that, for purposes of recovery as culture isolates, mutant as well as wild-type conidia are lost during the period of incubation and filtration but that wild-type conidia are eliminated much more rapidly. Certain mutant types are more difficult to recover from synthetic mixtures than are others. Favorable aspects of the method and possible reasons for the loss of mutant conidia are discussed.

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[1] Beadle, G. W., and Tatum, E. L., *Am. J. Bot.*, **32**, 678–686 (1945).

[2] Bowne, S. W., M.S. Thesis, Cornell University (1953).

[3] Fries, Nils, *Nature*, **159**, 199 (1947).

[4] Lein, J., Mitchell, H. K., and Houlahan, M. B., PROC. NATL. ACAD. SCI., **34**, 435–442 (1948).

[5] Tatum, E. L., Barratt, R. W., and Cutter, V. M., Jr., *Science*, **109**, 509–511 (1949).

[6] Tatum, E. L., Barratt, R. W., Fries, Nils, and Bonner, D., *Am. J. Bot.*, **37**, 38–46 (1950).

[7] Woodward, V. W., De Zeeuw, J. R., and Srb, A. M., *Genetics*, **37**, 637 (1952).

### BIBLIOGRAPHY

Beadle, G. W., and Tatum, E. L., "Neurospora. II. Methods of Producing and Detecting Mutations Concerned with Nutritional Requirements," *Am. J. Bot.*, **32**, 678–686 (1945).

Bowne, S. W., "The Relation of Heterotrophy to Survival of Conidia of *Neurospora crassa* Under Starvation Conditions," M.S. Thesis, Cornell, University, 1953.

Fries, Nils, 1947. "Experiments with Different Methods of Isolating Physiological Mutations of Filamentous Fungi," *Nature*, **159**, 199 (1947).

Lein, J., Mitchell, H. K., and Houlahan, M. B., "A Method for Selection of Biochemical Mutants of Neurospora," PROC. NATL ACAD. SCI., **34** 435–442 (1948).

Tatum, E. L., Barrartt, R. W., and Cutter, V. M., Jr., "Chemical Induction of Colonial Paramorphs in Neurospora and Syncephalastrum," *Science*, **109**, 509–511 (1949).

Tatum, E. L., Barratt, R. W., Fries, Nils, and Bonner, D., "Biochemical Mutant Strains of Neurospora Produced by Physical and Chemical Treatment," *Am. J. Bot.*, **37**, 38–46 (1950).

Woodward, V. W., De Zeeuw, J. R., and Srb, A. M., "Selection of Particular Biochemical mutants in Neurospora from Differentially Germinated Conidial Filtrates," *Genetics*, **37**, 637 (1952).

## *ON THE PLANCHEREL FORMULA FOR THE RIGHT-INVARIANT FUNCTIONS ON A SEMISIMPLE LIE GROUP*

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Let $G$ be a connected semisimple Lie group and $K$ the complete inverse image in $G$ of a maximal compact subgroup of its adjoint group. Let $\mu$ denote the invariant measure on the space $G/K$ of all cosets $xK(x \in G)$. We construct the space $L_2(G/K)$ with respect to $\mu$ and consider the problem of the complete reduction of the natural representation $\sigma$ of $G$ on $L_2(G/K)$. Let $D$ be any subgroup contained in the center

of $G$. Then $D \subset K$ and so if we replace $G$ by $G/D$ our problem is not affected in any way. Hence we may assume that the center of $G$ is finite and $K$ is compact. We can now regard $L_2(G/K)$ as a closed subspace of $L_2(G)$ consisting of those functions $f$ for which $f(x) = f(xu)$ $(x \in G, u \in K)$. Then $\sigma$ is the restriction of the left-regular representation of $G$ to $L_2(G/K)$. Let $dx$ denote the Haar measure on $G$. Put

$$(f, g) = \int_G \overline{f(x)} g(x)\, dx \qquad (f, g \in L_2(G))$$

where the bar denotes complex conjugate.

Let $\mathfrak{g}$ and $\mathfrak{k}_0$ be the Lie algebras of $G$ and $K$ respectively. In order to save space we shall adhere strictly to the notation of an earlier paper.[1] Thus any symbol should automatically be given the same meaning as there unless it is explicitly defined anew. Let $\eta$ be the anti-automorphism of $\mathfrak{B}$ over $R$ such that $\eta(X + (-1)^{1/2}Y) = -X + (-1)^{1/2}Y$ $(X, Y \in \mathfrak{g}_0)$. We denote by $\mathfrak{B}_H$ the set of those elements in $\mathfrak{B}$ which are left fixed by $\eta$. Let $\mathfrak{C}$ be the centralizer of $\mathfrak{k}$ in $\mathfrak{B}$. Put $\mathfrak{C}_0 = \mathfrak{C} \cap \mathfrak{B}_0$, $\mathfrak{C}_H = \mathfrak{C} \cap \mathfrak{B}_H$, and $\mathfrak{C}_{0,H} = \mathfrak{C}_0 \cap \mathfrak{C}_H$. (Here $\mathfrak{B}_0$ is the algebra generated over $R$ by $(1, \mathfrak{g}_0)$.) Let $\mathfrak{P}_d$ $(d \geq 0)$ denote the subspace of $\mathfrak{B}$ spanned over $C$ by the basic canonical elements[2] of degree $d$ formed out of a base for $\mathfrak{p}$. Let $\mathfrak{P} = \sum_{d \geq 0} \mathfrak{P}_d$. An element in $\mathfrak{P}$ is called homogeneous of degree $d$ if it lies in $\mathfrak{P}_d$. Let $l_+ = \dim_R \mathfrak{h}_{\mathfrak{p}_0} = \dim A_+$.

LEMMA 1. *The algebra $\mathfrak{C}^* = (\mathfrak{C} + \mathfrak{B}\mathfrak{k})/\mathfrak{B}\mathfrak{k}$ is abelian and there exist $l_+$ elements $z_i$ $1 \leq i \leq l_+$ in $\mathfrak{C}_{0,H}$ such that their residue-classes mod $\mathfrak{B}\mathfrak{k}$ generate $\mathfrak{C}^*$. $z_i$ may be chosen to be homogeneous elements in $\mathfrak{P} \cap \mathfrak{C}_{0,H}$.*

Let $C_c(G)$ denote the space of all complex-valued continuous functions $f$ on $G$ which vanish outside a compact set and $C_c^\infty(G)$ the subspace consisting of those $f$ which are indefinitely differentiable everywhere. Moreover let $C_c(G/K)$ and $I_c(G)$, respectively, be the subsets of those $f \in C_c(G)$ for which $f(xu) = f(x)$ and $f(uxu^{-1}) = f(x)$ $(x \in G, u \in K)$. Put $I_c(G/K) = I_c(G) \cap C_c(G/K)$ and denote the closures of $I_c(G)$ and $I_c(G/K)$ in $L_1(G)$ and $L_2(G/K)$ by $I_1(G)$ and $I_2(G/K)$ respectively. For any $f$ and $g$ in $L_1(G)$ we denote by $f*g$ their convolution and by $\sigma(f)$ the operator $\int f(x)\sigma(x)\, dx$. Then $f \to \sigma(f)$ is a continuous representation of $L_1(G)$ and it is clear that if $f \in I_1(G)$, $I_2(G/K)$ is invariant under $\sigma(f)$. Let $\tau(f)$ denote its restriction on $I_2(G/K)$. Then $f \to \tau(f)$ is a representation of the subalgebra $I_1(G)$ on $I_2(G/K)$.

LEMMA 2. *$\tau$ is an abelian representation of $I_1(G)$.*

Let $V$ denote the Garding subspace[3] of $L_2(G/K)$ with respect to $\sigma$ and $b \to \sigma(b)$ $(b \in \mathfrak{B})$ the corresponding representation of $\mathfrak{B}$ on $V$. It is obvious that for any $z \in \mathfrak{C}$, $\sigma(z)$ leaves $V_I = V \cap I_2(G/K)$ invariant. We denote by $\tau(z)$ the restriction of $\sigma(z)$ on $V_I$ $(z \in \mathfrak{C})$.

LEMMA 3. *If $z \in \mathfrak{C}_H$, $\tau(z)$ is an essentially hypermaximal operator on $I_2(G/K)$.*

For any $z \in \mathfrak{C}_H$ let $U(z)$ denote the Cayley transform of $\tau(z)$. Then $U(z)$ is a unitary operator on $I_2(G/K)$ and

$$U(z)(\sigma(z) + (-1)^{1/2}I)g = (\sigma(z) - (-1)^{1/2}I)g \qquad (g \in V_I)$$

where $I$ is the unit operator on $I_2(G/K)$. Put $U_i = U(z_i)$ $(1 \leq i \leq l_+)$ where $z_i$ are the elements defined in Lemma 1. Let $\mathfrak{A}$ be the smallest $C^*$-algebra[4] of operators

on $I_2(G/K)$ containing $\tau(f)(f \in I_1(G))$ and $U_i$ $1 \leq i \leq l_+$. One proves without difficulty that $\mathfrak{A}$ is abelian. Let $\Gamma$ be the spectrum of $\mathfrak{A}$. Then $\Gamma$ is a compact Hausdorff space. For any $\omega \in \Gamma$ we denote by $\xi_\omega$ the corresponding continuous character of $\mathfrak{A}$. Let $\lambda$ and $\rho$, respectively, denote the left- and the right-regular representations of $G$ on $L_1(G)$. Put

$$E = \int_K \lambda(u)\rho(u)\, du$$

where $du$ is the normalized Haar measure on $K$ so that $\int_K du = 1$. Then $E$ is a projection from $L_1(G)$ to $I_1(G)$ and $||Ef||_1 \leq ||f||_1$ $(f \in L_1(G))$ where $||\ \ ||_1$ denotes the norm in $L_1(G)$. Now set

$$\xi_\omega(f) = \xi_\omega(\tau(Ef)) \qquad (f \in L_1(G)).$$

Then if $\tilde{f}(x) = \overline{f(x^{-1})}(f \in L_1(G), x \in G)$ it is easy to verify that $|\xi_\omega(f)| \leq ||f||_1$ and $\xi_\omega(\tilde{f} * f)$ is real and non-negative $(f \in L_1(G))$. Let $\Gamma_0$ denote the set of all $\omega \in \Gamma$ such that $\xi_\omega$ vanishes identically on $L_1(G)$. Then it follows from well-known results[5] that if $\omega \in \Gamma_0$ there exists a unitary representation $\pi$ of $G$ on a Hilbert space $\mathfrak{H}$ and a vector $\psi \in \mathfrak{H}$ such that

(i) $|\psi| = 1$ and $\xi_\omega(f) = \int_G f(x)(\psi, \pi(x)\psi)\, dx$ $(f \in L_1(G))$.

(ii) The space spanned by $\pi(x)\psi (x \in G)$ is dense in $\mathfrak{H}$.

Here $(\psi_1, \psi_2)$ denotes the scalar product and $|\psi|$ the norm in $\mathfrak{H}$. It is easy to verify that $\xi_\omega(\lambda(u)f) = \xi_\omega(\rho(u)f) = \xi_\omega(f)(f \in L_1(G), u \in K)$. From this one can prove that $\pi(u)\psi = \psi(u \in K)$ and $\pi$ is irreducible. Moreover the choice of $\psi$ is unique, apart from a unimodular factor, and $\psi$ is well-behaved[6] under $\pi$. Put $\xi_\omega(b) = (\psi, \pi(b)\psi)(b \in \mathfrak{B})$. Then it is not difficult to show that

$$\xi_\omega(U_i) = (\xi_\omega(z_i) - (-1)^{1/2})(\xi_\omega(z_i) + (-1)^{1/2})^{-1} \qquad 1 \leq i \leq l_+.$$

Now let $\omega_1$, $\omega_2$ be two points in $\Gamma - \Gamma_0$ and $\pi_1$, $\pi_2$ the corresponding irreducible representations. Then if $\xi_{\omega_1}(U_i) = \xi_{\omega_2}(U_i)$, $1 \leq i \leq l_+$, it follows from Lemma 1 that $\xi_{\omega_1}(z) = \xi_{\omega_2}(z)(z \in \mathfrak{Z})$ and therefore[7] $\pi_1$ and $\pi_2$ are equivalent. Hence $\xi_{\omega_1}(f) = \xi_{\omega_2}(f)$ for all $f \in L_1(G)$ and so $\omega_1 = \omega_2$. Let $\mathfrak{E}$ denote the set of all equivalence classes of irreducible unitary representations $\pi$ of $G$ such that the trivial representation of $K$ occurs in the reduction of $\pi$. Then if $\omega \in \Gamma - \Gamma_0$ and $\alpha(\omega)$ is the class of the representation $\pi$ defined above for $\omega$, $\omega \to \alpha(\omega)$ is a 1-1 mapping of $\Gamma - \Gamma_0$ onto a subset $\mathfrak{E}_0$ of $\mathfrak{E}$. We identify $\Gamma - \Gamma_0$ with $\mathfrak{E}_0$ under this mapping.

On the other hand it is well known that there exists a unique projection-valued (regular) measure $dP_\omega$ on the class of all Borel subsets of $\Gamma$ such that

$$A = \int_\Gamma \xi_\omega(A) dP_\omega$$

for every operator $A$ in $\mathfrak{A}$. Since $\Gamma_0$ is clearly compact, it is Borel measurable and if $P_{\Gamma_0}$ is the corresponding projection it is obvious that $\tau(f)P_{\Gamma_0} = 0$ for all $f \in I_1(G)$. Moreover since it is possible to choose a sequence $f_n \in I_1(G)$ such that $\tau(f_n)g \to g$ for every $g \in I_2(G/K)$, it follows that $P_{\Gamma_0} = 0$. From this we can conclude that $\Gamma_0$ contains no interior point and therefore if $\omega_0 \in \Gamma_0$ there exists a sequence $\omega_n \in \Gamma - \Gamma_0$ such that $\omega_n \to \omega_0$ in $\Gamma$. Furthermore

$$A = \int_{\mathfrak{E}_0} \xi_\omega(A) dP_\omega \qquad (A \in \mathfrak{A}).$$

For any $\omega \in \mathfrak{E}_0$ let $Q(\omega)$ denote the point $(\xi_\omega(z_1), \ldots, \xi_\omega(z_{l_+}))$ in the real Euclidean space of dimension $l_+$.

LEMMA 4. *Let $\omega_n$ be a sequence in $\mathfrak{E}_0$ which converges in $\Gamma$ to a point $\omega_0 \in \Gamma_0$. Then the sequence $Q(\omega_n)$ diverges to infinity.*

In view of the above-mentioned relationship between $\xi_\omega(U_i)$ and $\xi_\omega(z_i)$ for $\omega$ in $\mathfrak{E}_0$, it follows that $\xi_{\omega_n}(U_i) \to 1$ for at least one value of $i$ $(1 \leq i \leq l_+)$. This proves that $\xi_{\omega_0}(U_i) = 1$ for some $i$ if and only if $\omega_0 \in \Gamma_0$.

Now let $\omega_1$, $\omega_2$ be two points in $\Gamma$ such that $\xi_{\omega_1}(U_i) = \xi_{\omega_2}(U_i)$, $i = 1, \ldots, l_+$. Suppose $\omega_1 \in \Gamma_0$. Then $\xi_{\omega_1}(U_i) = 1$ for some $i$. Therefore since $\xi_{\omega_2}(U_i) = \xi_{\omega_1}(U_i) = 1$ it follows that $\omega_2 \in \Gamma_0$. Hence $\omega_1$, $\omega_2$ are both in $\Gamma_0$ and so $\xi_{\omega_1}$, $\xi_{\omega_2}$ are identically zero on $L_1(G)$. It is then clear that $\omega_1 = \omega_2$. On the other hand if $\omega_1 \notin \Gamma_0$, we conclude in the same way that $\omega_2 \notin \Gamma_0$ and therefore again in view of our earlier remarks $\omega_1 = \omega_2$. This shows that $\xi_\omega(U_i)$ $1 \leq i \leq l_+$ is a separating set of continuous functions on $\Gamma$. Therefore from the Stone-Wierstrass theorem we can conclude that $\mathfrak{A}$ is the smallest $C^*$-algebra containing the operators $U_i$, $1 \leq i \leq l_+$. Then it follows from Lemma 4 that $\omega \to Q(\omega)$ is a topological mapping of $\mathfrak{E}_0$ onto a subset $Q(\mathfrak{E}_0)$ of the Euclidean space. Let $\tau_i$ denote the unique hypermaximal extension of $\tau(z_i)$, $1 \leq i \leq l_+$, and let $dP_\lambda$ denote the projection-valued Borel measure on $Q(\mathfrak{E}_0)$ corresponding to $dP_\omega$ on $\mathfrak{E}_0$. Then it is clear from the above remarks that $Q(\mathfrak{E}_0)$ is the spectrum of the abelian set $\tau_i$, $1 \leq i \leq l_+$, of hypermaximal operators on $I_2(G/K)$ and

$$\tau_i = \int_{Q(\mathfrak{E}_0)} \lambda_i \, dP_\lambda \qquad 1 \leq i \leq l_+$$

where $\lambda_i(Q(\omega)) = \xi_\omega(z_i)$ $(\omega \in \mathfrak{E}_0)$.

On the other hand it is not difficult to show that there exists a uniquely determined (regular) Borel measure $d\omega$ on $\mathfrak{E}_0 = \Gamma - \Gamma_0$ such that

$$d(f, P_\omega g) = \overline{\xi_\omega(f)}\xi_\omega(g) \, d\omega \qquad (f, g \in I_c(G/K), \omega \in \mathfrak{E}_0).$$

From this one can prove that

$$\int |f(x)|^2 dx = \int_{\mathfrak{E}_0} \xi_\omega(\check{f} * f) \, d\omega \qquad (f \in C_c(G/K)).$$

Let $\pi$ be any unitary representation in the class $\omega \in \mathfrak{E}_0$. Then if $N_\omega(f)$ denotes the square of the Hilbert-Schmidt norm of the operator $\int f(x)\pi(x) \, dx$ $(f \in C_c(G))$ it is easy to verify that $\xi_\omega(\check{f} * f) = N_\omega(f)$ $(f \in C_c(G/K))$. Hence we have the Plancherel formula

$$\int |f(x)|^2 \, dx = \int_{\mathfrak{E}_0} N_\omega(f) \, d\omega \qquad (f \in C_c(G/K)).$$

Our results may therefore be summarized in the following theorem.

THEOREM. *Let $F$ be the spectrum of the abelian family of hypermaximal operators $\tau_i$, $1 \leq i \leq l_+$, regarded as a subset of the real Euclidean space $E$ of dimension $l_+$. Let $P_\lambda$ denote the corresponding resolution of the identity so that*

$$\tau_i = \int_F \lambda_i \, dP_\lambda \qquad (1 \leq i \leq l_+)$$

*where $\lambda_i$ is the $i$th coordinate of any point $\lambda \in E$. Then there exists a 1-1 mapping $\lambda \to \omega(\lambda)$ of $F$ on a subset $\mathfrak{E}_0$ of $\mathfrak{E}$ with the following property.*

*If $\pi$ is any representation in $\omega(\lambda)$ and $\psi$ is the (essentially unique) vector in its representation space such that $|\psi| = 1$ and $\pi(u)\psi = \psi(u \in K)$, then*

$$\lambda_i = (\psi, \pi(z_i)\psi) \qquad 1 \leq i \leq l_+.$$

*Put* $\varphi_\lambda(x) = (\psi, \pi(x)\psi)(x \in G)$. *Then there exists a uniquely determined (regular) Borel measure* $d\lambda$ *on* $F$ *such that*

$$d(f, P_\lambda f) = |\int f(x)\varphi_\lambda(x)\, dx|^2\, d\lambda \qquad (f \in I_c(G/K)).$$

*Moreover we have the following Plancherel formula*

$$\int |f(x)|^2\, dx = \int_F N_{\omega(\lambda)}(f)\, d\lambda \qquad (f \in C_c(G/K)).$$

Let $W$ denote the group of all those linear mappings $s$ of $\mathfrak{h}_{\mathfrak{p}_0}$ into itself for which we can find an element $u \in K$ such that $sH = Ad(u)H (H \in \mathfrak{h}_{\mathfrak{p}_0})$. Then $W$ is a finite group. Let $I_c(\mathfrak{h}_{\mathfrak{p}_0})$ denote the set of all continuous functions $f$ on $\mathfrak{h}_{\mathfrak{p}_0}$ vanishing outside a compact set such that $f(H) = f(sH)$ $(s \in W, H \in \mathfrak{h}_{\mathfrak{p}_0})$. For any $f \in I_c(G/K)$ put $f_0(H) = f(\exp H)$ $(H \in \mathfrak{h}_{\mathfrak{p}_0})$. Since $G = KA_+K$ it is easy to show that $f \to f_0$ is a 1-1 mapping of $I_c(G/K)$ onto $I_c(\mathfrak{h}_{\mathfrak{p}_0})$ and

$$\int |f(x)|^2\, dx = \int_{\mathfrak{h}_{\mathfrak{p}_0}} |f_0(H)|^2 \Delta_+(H)\, dH$$

where $\Delta_+(H) = \prod_{\alpha \in P\lambda} |e^{\alpha(H)} - e^{-\alpha(H)}|$ and $dH$ is the Euclidean measure on $\mathfrak{h}_{\mathfrak{p}_0}$. Let $L_2(\mathfrak{h}_{\mathfrak{p}_0})$ denote the Hilbert space of all functions on $\mathfrak{h}_{\mathfrak{p}_0}$ which are square-integrable with respect to the measure $\Delta_+(H)\, dH$. Let $I_2(\mathfrak{h}_{\mathfrak{p}_0})$ denote the closure of $I_c(\mathfrak{h}_{\mathfrak{p}_0})$ in $L_2(\mathfrak{h}_{\mathfrak{p}_0})$. Then the above mapping $f \to f_0$ can be extended uniquely to a unitary isomorphism of $I_2(G/K)$ with $I_2(\mathfrak{h}_{\mathfrak{p}_0})$. Under this isomorphism the operators $\tau(z_i)$ $1 \leq i \leq l_+$ go over into certain differential operators $D_i$ whose coefficients are meromorphic functions on $\mathfrak{h}_{\mathfrak{p}_0}$. $F$ and $dP_\lambda$ of our theorem may now, respectively, be interpreted as the spectrum and the spectral measure of the family $D_i$ $1 \leq i \leq l_+$ and the above Plancherel formula then becomes the "Parseval formula" corresponding to the problem of expanding a given function in $I_2(\mathfrak{h}_{\mathfrak{p}_0})$, in terms of eigen-functions of the family $D_i$ $1 \leq i \leq l_+$ of differential operators.

[1] *Trans. Am. Math. Soc.*, **75**, 185–243 (1953). See especially §2 and §12 for notation. We shall refer to this paper as *RI*.

[2] See *RI*, p. 193.

[3] See §4 of *RI* for the definition of the Garding subspace.

[4] A C*-algebra is an algebra $\mathfrak{A}$ of bounded operators on a Hilbert space such that (i) the unit operator is in $\mathfrak{A}$ (ii) the adjoint of any operator in $\mathfrak{A}$ is also in $\mathfrak{A}$ (iii) $\mathfrak{A}$ is closed under uniform topology.

[5] I. Gelfand and D. Raikov, *Recueil Math. (Moscow)*, **13**, 316 (1943).

[6] See Theorem 6, §11 of *RI*.

[7] See PROC. NATL. ACAD. SCI., **37**, 170–173 (1951); Theorem 7.

# *PROOF OF A CONJECTURE OF STEINHAUS*

By Henry Helson*

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Steinhaus has conjectured[1] the following theorem:
Suppose a trigonometric series

$$\sum_{-\infty}^{\infty} a_n e^{inx}$$

is given with the property that the partial sums

$$\sum_{-N}^{N} a_n e^{inx}$$

are non-negative for all $x$ and all sufficiently large $N$. Then $a_n$ tends to zero as $|n|$ tends to infinity.

Instead of supposing the partial sums are non-negative, one may assume only that

$$\int_0^{2\pi} \Big| \sum_{-N}^{N} a_n e^{inx} \Big| dx$$

is bounded as $N$ tends to infinity, and hope to conclude as before that the coefficients tend to zero.

We shall prove the conjecture in the second form, embracing the first. Partial results have been published by Sidon[2] and by Erdös.[3] Recently Salem and Zygmund have proved a theorem about the "average" behavior of the coefficients which is not a consequence of the theorem proved here.[4]

Let us assume then that

$$1^\circ \quad |a_{n_j}| \geqslant \epsilon > 0 \text{ for a sequence } n_j$$

tending to infinity, and

$$2^\circ \quad \int^{2\pi} \Big| \sum_{-N}^{N} a_n e^{inx} \Big| dx$$

is bounded as $N$ tends to infinity. We shall derive a contradiction.

The second condition implies that the $a_n$ are the Fourier-Stieltjes coefficients of a function $\mu(x)$ of bounded variation:

$$a_n = \frac{1}{2\pi} \int_0^{2\pi} e^{-inx}\, d\mu(x).$$

Now the measures written symbolically as

$$e^{-in_j x}\, d\mu(x)$$

all have the same total variation as $\mu(x)$. By the Helly theorem there is a function $\nu(x)$ of bounded variation and a subsequence of $j$ such that

$$\int_0^{2\pi} \varphi(x)\, d\gamma(x) = \lim \int_0^{2\pi} \varphi(x) e^{-in_j x}\, d\mu(x)$$

for every continuous periodic function $\varphi(x)$. If $\mu(x)$ is replaced by its singular part $\mu_s(x)$ the limit is unchanged, since the Fourier coefficients of an absolutely continuous measure tend to zero. Let $\varphi(x)$ range over the continuous functions vanishing outside a given arc of the circle, and bounded in modulus by one. The least upper bound of the modulus of the left side is exactly the variation of $\gamma(x)$ over that arc (not counting the end-points), and this does not exceed the variation of $\mu_s(x)$ over the arc. Consequently the derivative of $\gamma(x)$ is zero almost everywhere, and $\gamma(x)$ is a singular function. Moreover $\gamma(x)$ is not constant, for its central coefficient is the limit of certain $a_{n_j}$ (as we see by taking $\varphi(x)$ a constant function), which has to be different from zero by the first condition.

On the other hand we can form the measures

$$e^{-in_j x} \sum_{-n_j}^{n_j} a_n e^{inx}\, dx$$

for those $j$ used to define $\gamma(x)$. By the second condition the sequence is uniformly of bounded variation, and so by the Helly theorem contains a subsequence converging weakly to a function $\gamma'(x)$. The Fourier-Stieltjes coefficients of $\gamma'(x)$ evidently vanish for positive indices, and coincide with the coefficients of $\gamma(x)$ on the left. By a theorem of F. and M. Riesz,[5] a function of bounded variation whose coefficients all vanish on one side is absolutely continuous. Hence $\gamma'(x)$ and $\gamma(x) - \gamma'(x)$ are absolutely continuous, and the same must be true of $\gamma(x)$ itself.

But $\gamma(x)$ has been shown to be a non-trivial singular function. This contradiction proves that the two conditions on the series are incompatible, and the theorem is proved.

Evidently the proof holds if the second condition is assumed to hold only as $N$ tends to infinity through the sequence of numbers $n_j$. However, the following question is open: does Condition 2 imply that the measure $d\mu(x)$ is absolutely continuous? An affirmative answer would, of course, contain the Steinhaus theorem.

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[1] I do not know the occasion of the original statement. The problem was suggested to me by Professor R. Salem.

[2] "Über die Fourier-Koeffizienten einer stetigen Funktion von beschränkter Schwankung," *Acta Szeged*, **2**, 43–46 (1924).

[3] "On a conjecture of Steinhaus," *Univ. Nac. Tucamán Revista A*, **7**, 217–220 (1940).

[4] The theorem of Salem and Zygmund asserts that under the hypothesis, the coefficients satisfy $(|a_1| + \ldots + |a_n|)/n = 0(1/\log n)$.

[5] "Über die Randwerte einer analytischen Funktion," *Quatrième Congrès des math. scandinaves*, (1916), pp. 27–44.

# *ON CERTAIN INEQUALITIES RELATING THE BETTI NUMBERS OF A MANIFOLD AND ITS SUBSETS**

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It is the purpose of this note to show how, by decomposition of certain well-known exact sequences and use of generalized forms of the Poincaré duality theorem, one may easily obtain, in very general form, relations between the Betti numbers of a closed subset of a closed orientable generalized manifold and those of its complement. Among these are (1) inequalities established by Morse[1] for certain differentiable manifolds embedded in Euclidean spaces, (2) an inequality recently found by P. A. White,[2] and (3) a relation given by L. Pontrjagin[3] in 1928 and recently generalized by one of the present authors[4] (using other methods, however). Throughout we use Čech homology (augmented except in the case of the Poincaré duality relations) over an arbitrary field.

Theorem 1. *Let $M$ be a closed subset of a compact Hausdorff space $S$. Then the following relations hold among the Betti numbers:*

$$\begin{aligned} p^r(M) &\leqq p^r(S) + p^{r+1}(S, M) \\ p^r(S) &\leqq p^r(M) + p^r(S, M) \\ p^{r+1}(S, M) &\leqq p^r(M) + p^{r+1}(S). \end{aligned} \tag{1}$$

*Proof:* Consider the homology sequence of the compact pair $(S, M)$:

$$\cdots \xrightarrow{j} H^{r+1}(S, M) \xrightarrow{\partial} H^r(M) \xrightarrow{i} H^r(S) \xrightarrow{j} H^r(S, M) \xrightarrow{\partial} \cdots.$$

Since this sequence is exact and the coefficient group is a field, the group $H^r(M)$ may be expressed as the direct sum of the image $H_0{}^r(M)$ of $H^{r+1}(S, M)$ under $\partial$ and a subgroup $H_1{}^r(M)$ which is isomorphic to the image of $H^r(M)$ itself under the mapping $i$; thus

$$H^r(M) = H_0{}^r(M) + H_1{}^r(M).$$

A similar decomposition may be made of each of the other groups appearing in the sequence, and we may rewrite the sequence as follows:

$$\cdots \xrightarrow{j} H_0{}^{r+1}(S, M) + H_1{}^{r+1}(S, M) \xrightarrow{\partial} H_0{}^r(M) + H_1{}^r(M) \xrightarrow{i}$$
$$H_0{}^r(S) + H_1{}^r(S) \xrightarrow{j} H_0{}^r(S, M) + H_1{}^r(S, M) \xrightarrow{\partial} \cdots,$$

where each subgroup immediately preceding an arrow is isomorphic to the subgroup immediately following; e.g., $H_1{}^{r+1}(S, M) \cong H_0{}^r(M)$, $H_1{}^r(S) \cong H_0{}^r(S, M)$.

Consequently we have

$$H^r(M) \cong H_1{}^{r+1}(S, M) + H_0{}^r(S) \tilde{\subset} H^{r+1}(S, M) + H^r(S),$$

where the symbol "$\tilde{\subset}$" means "is isomorphic to a subgroup of." Thus it follows that $p^r(M) \leqq p^{r+1}(S, M) + p^r(S)$. And in a similar fashion the second and third inequalities of (1) are shown to hold.

THEOREM 2. *If $M$ is a closed subset of an orientable $n$-gcm,*[5] *then the following relations hold:*

$$\begin{aligned} p^r(M) &\leqq p^r(S) + p^{n-r-1}(S - M) \\ p^r(S) &\leqq p^r(M) + p^{n-r}(S - M) \\ p^{n-r-1}(S - M) &\leqq p^r(M) + p^{n-r-1}(S), \end{aligned} \tag{2}$$

*where $p^k(S - M)$ denotes the dimension of the $k$th compact homology group*[6] *of $S - M$.*

*Proof:* Using the following generalized forms[7] of the Poincaré duality relation for $n$-gcm's,

$$\begin{aligned} p^{r+1}(S, M) &= p^{n-r-1}(S - M) \\ p^{r+1}(S) &= p^{n-r-1}(S), \end{aligned}$$

the theorem follows directly from Theorem 1.

The third of the inequalities (2) was obtained by Morse[1] for cases where $M$ and $S$ satisfy certain special conditions.

If, in the first of the inequalities (2), we let $r = n - 1$, we get $p^{n-1}(M) \leqq p^{n-1}(S) + p^0(S - M)$. Since the Betti numbers employed in the statement of the Poincaré duality are of the "non-augmented" type, $p^0(S - M)$ is the number of components of $S - M$. Denoting the latter by $m$, we get $p^{n-1}(M) \leqq p^{n-1}(S) + m$. Actually, as shown by White,[2] the upper bound so obtained for $p^{n-1}(M)$ can be decreased by 1 if we assume $M$ a *proper* subset of $S$ (a fact which has added interest since $m - 1$ is a *lower* bound for $p^{n-1}(M)$, as shown in $TM^5$, p. 213). This is at once evident if we recall the origin of the inequality in the relation $H^{n-1}(M) = H_1{}^n(S, M) + H_0{}^{n-1}(S)$. For if $M$ is a proper subset of $S$ then $H^n(M) = 0$ so that $j: H^n(S) \to H_0{}^n(S, M)$ is an isomorphism; and $H^n(S)$ having dimension 1 we get dim $H_1{}^n(S, M)$ = dim $H^n(S, M)$ − dim $H_0{}^n(S, M) = p^n(S, M) - 1 = p_0(S - M) - 1 = m - 1$.

THEOREM 3. *Let $M$ be the boundary of an open subset $U$ of an orientable $n$-gcm $S$. Then*

$$p^r(M) \leqq p^r(\bar{U}) + p^{n-r-1}(U). \tag{3}$$

*And if $U$ is ulc$^{n-1}$, then*

$$p^r(M) \leqq p^r(\bar{U}) + p^{n-r-1}(\bar{U}). \tag{4}$$

*Proof:* The relation (3) may be obtained from analysis of the exact sequence of the pair $(\bar{U}, M)$, analogous to the analysis for the pair $(S, M)$ in the proof of Theorem 1. If $U$ is ulc$^{n-1}$, then $p^{n-r-1}(U) = p^{n-r-1}(\bar{U})$, and (4) follows from (3).

Relation (4) was obtained by Morse[1] under stronger assumptions on the sets involved.

DEFINITION. *If $S$ is an orientable $n$-gcm and $M$ a closed subset of $S$, then $q^r(M)$ denotes the dimension of the vector space of $r$-cycles of $M$ that bound in $S$, reduced modulo the subspace of cycles that bound in $M$. A number $q^r(S - M)$ is similarly defined in terms of compact cycles of $S - M$.*

The relation referred to in (3) of the opening paragraph above is embodied in the following theorem:

THEOREM 4. *Let $M$ be a closed subset of an orientable $n$-gcm $S$. Then the following relation holds:*

$$p^{n-r-1}(S - M) = q^r(M) + p^{n-r-1}(S) - [p^{r+1}(M) - q^{r+1}(M)]. \quad (5)$$

*Proof:* As in the proof of Theorem 1, we have:

$$H^{r+1}(S, M) \cong H_1{}^{r+1}(S) + H_0{}^r(M).$$

And from the analogous direct sum decomposition for $H^{r+1}(S)$ we have that $H_1{}^{r+1}(S) \cong H^{r+1}(S) - H_0{}^{r+1}(S) \cong H^{r+1}(S) - H_1{}^{r+1}(M)$. Hence we may write

$$H^{r+1}(S, M) = [H^{r+1}(S) - H_1{}^{r+1}(M)] + H_0{}^r(M), \quad (6)$$

and, in dimensional terms,

$$p^{r+1}(S, M) = p^{r+1}(S) - \dim H_1{}^{r+1}(M) + \dim H_0{}^r(M). \quad (7)$$

However, dim $H_0{}^r(M)$ is evidently $q^r(M)$. Also, since $H_1{}^{r+1}(M) \cong H^{r+1}(M) - H_0{}^{r+1}(M)$, we have dim $H_1{}^{r+1}(M) = p^{r+1}(M) - q^{r+1}(M)$. Substituting in (7) gives

$$p^{r+1}(S, M) = p^{r+1}(S) - [p^{r+1}(M) - q^{r+1}(M)] + q^r(M). \quad (8)$$

The transformation of (8) into (5) is now accomplished by means of the generalized form of the Poincaré duality relation stated in the proof of Theorem 2.

* Presented to the American Mathematical Society April 23, 1953. Work on this paper was sponsored by OOR, U. S. Army, under Contract No. DA-20-018-ORD-12738.

[1] Morse, M., these PROCEEDINGS, **38,** 247–258 (1952).

[2] White, P. A., *Proc. Am. Math. Soc.*, **3,** 488–498 (1952).

[3] Pontrjagin, L., *Nachr. Ges. Wiss. Göttingen,* **7,** (1927), pp. 446–456.

[4] Wilder, R. L., *Proc. Intern. Congr. Mathematicians 1950,* **1,** 530–531 (1952).

[5] Wilder, R. L., *Topology of Manifolds,* Am. Math. Soc. Coll. Pub., Vol. 32, 1949; referred to hereafter as *TM*. The classical types of closed $n$-dimensional manifolds are special cases of the *n-gcm;* the latter is generally not susceptible to decomposition into "cells."

[6] See *TM,* p. 246.

[7] See *TM,* pp. 253 and 259.

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## *THE PHASE CHARACTERISTICS OF THE EAR**

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*Communicated January 12, 1954*

One of the ways in which the properties of a sound-receiving system are revealed is through the phase changes that the system introduces into the signals that it transmits. In earlier experiments we have dealt with the phase changes produced by the middle ear apparatus.[1, 2] The present experiments extend this study to a consideration of the phase changes produced by the ear as a whole.

These experiments have been carried out on the cat, and by use of the electrical potentials of the cochlea as an indicator of the ear's activity in response to sounds. Specifically, the measurements represent the phase relation between the pressure waves of sound stimulating the ear and the resulting waves of cochlear potential.

*Procedure.*—The experimental procedure was similar to the one used in our earlier experiments, just referred to. The animals were deeply anesthetized with diallyl-barbituric acid injected intraperitoneally. The auditory bulla was approached by the mediolateral route and opened to give access to the round window. A platinum foil electrode was placed in contact with the round window membrane and an indifferent electrode was inserted into the masseter muscle. Sometimes a second foil electrode was applied to the bony surface of the cochlear capsule at its apical end and readings were made at both basal and apical positions.

In most of the animals the further surgical procedures were carried out in two stages. In the first stage the pinna and the membranous portion of the external auditory meatus were removed so as to expose the drum membrane to clear view. A sound cannula leading from a loudspeaker was inserted into the meatal opening and sealed there with wax. This sound cannula carried a probe tube connected with a condenser microphone. The probe tube entered the cannula through its side and then ran concentrically with it to end at its orifice 2 to 3 mm. from the drum membrane.

In the second stage of the surgical procedure the drum membrane, malleus, and incus were removed and the sound cannula was sealed over the oval window so as to enclose the stapes without touching it.

As in our earlier studies, the phase measurements were made by a wave-matching method in which the two waves were presented together on the face of a dual-beam cathode-ray oscillograph. The sound applied to the ear was picked up by the acoustic probe and its associated microphone and the resulting currents were amplified and conducted to one beam of the oscillograph. The cochlear potentials were amplified and conducted to the other beam of the oscillograph. The first of these channels also contained a phase changer that was set to compensate for any phase differences introduced by circuit elements of the two channels. These settings usually differed for every frequency, and an important variable was the probe and microphone combination.

The phase calibration of our probe and microphone was obtained in the following way. Another microphone was first calibrated by an indirect method on the assumption that its phase response follows that of a simple resonator. A pressure calibration was obtained by the reciprocity method, the response curve of a simple resonator was fitted to this pressure curve, and the phase variations were then derived.[3] A comparison of the results of this procedure with those obtained by Wiener[4, 5] for the same type of microphone (Western Electric Type 640 AA) by a direct method proves its suitability for our present purposes: the calculated and measured values for Wiener's microphone were in agreement on the average within 3.8 deg. of phase and the maximum divergence was 8 deg. These variations are of the order of magnitude of errors of measurement inherent in our experimental situation, which we have found to be about 5 deg. The phase calibration of this microphone was then transferred to our probe tube and microphone combination by the use of a special calibration chamber with a volume (including the volume of the probe and microphone combination) of 0.32 cc.

The experiments were carried out on 10 cat ears, and included 35 determinations of phase relations between cochlear potentials and the applied sounds, of which 19 were with the middle ear apparatus intact and 16 were with the drum membrane,

malleus, and incus removed. Observations were made for a number of tones over the frequency range from 100 to 10,000 cycles.

*Results.*—It was first established that the phase changes produced by the ear at any given frequency are independent of the stimulus intensity at all levels up to the point where the ear begins to overload. As overloading enters, the phase changes depart from their values at lower levels and become erratic. In the experiments reported here the stimulus intensity was kept to a moderate level, and for every reading the tone was delivered at two or more intensities so as to provide a check on the linearity of the responses.

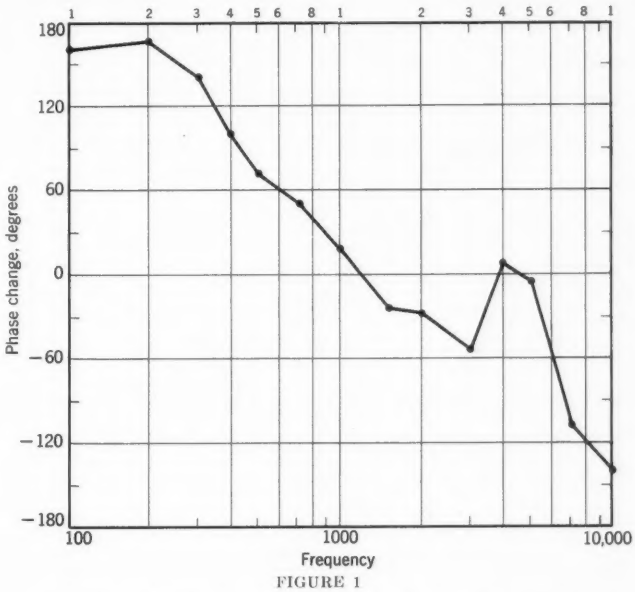


FIGURE 1

Phase changes introduced by the ear as a whole. The measurements represent differences between the positive peak of sound pressure applied at the drum membrane and the positive peak of electrical potentials recorded from the cat's round window membrane. Positive values of phase indicate a lag in the transmitted waves.

A series of measurements with the middle ear intact and with the recording electrode on the round window membrane is shown in figure 1. In this figure, as in others to follow, the stimulating frequencies are represented along the abscissa and the phase changes from stimulus to response are represented along the ordinate. Positive values of phase mean that the electrical response of the ear is lagging behind the stimulus, and negative values mean that this response is advanced in phase with respect to the stimulus. More concretely, a positive value of phase signifies that the positive peak of the electrical potential is lagging with respect to the positive peak of the sound pressure.

It is evident that there is a fairly progressive change from phase lag to phase advance as the stimulating frequency is raised from 100 to 10,000 cycles. The

greatest lags are for the low tones, for which they approach 180 deg., and then the lag diminishes regularly as the frequency rises, until zero phase is passed through around 1200 cycles. Thereafter the change is generally toward an increasingly advanced phase, except for a reversal at 4000 and 5000 cycles.

The general pattern of phase change as a function of frequency as shown in this figure was observed for all the ears studied, but sometimes with less regularity. Variations were especially prominent in ears with signs of having suffered from middle ear disease. The larger variations were found for the high frequencies, those above 5000 cycles.

In some of the experiments curare was administered through the femoral vein in amounts sufficient to depress general muscle activity, after which the animal was

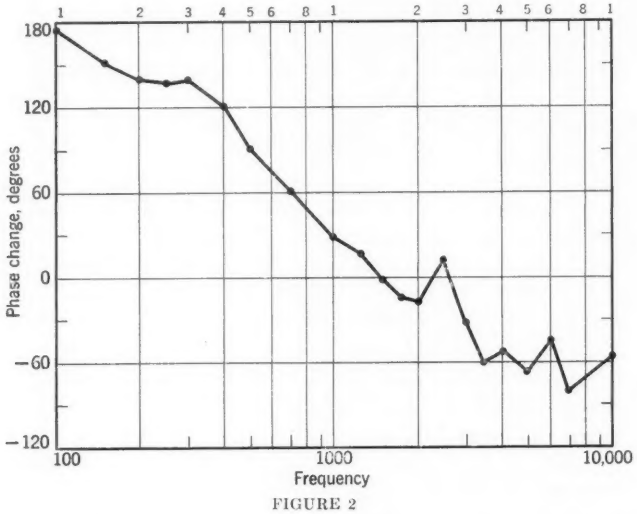


FIGURE 2

Phase changes produced by the inner ear. The drum membrane and two outer ossicles were removed and the sounds were applied directly to the footplate of the stapes. The recording electrode was at the round window.

maintained by artificial respiration. This was done in order to exclude any modifications of sound transmission by contractions of the middle ear muscles. We found no outstanding changes of the phase pattern as a result of such curarization. The curves were perhaps a little more regular, especially those obtained from ears showing scarred and thickened membranes as a result of former middle ear disease, but the general form of the phase pattern was unaltered.

In all the ears, and especially in those showing irregularities, the pattern was considerably simplified after removal of the peripheral portion of the middle ear and application of the sounds directly to the stapes. Some of the results obtained by this method, and with the electrode on the round window membrane, are represented in figure 2. The phase changes are similar to those shown by the intact ear for the low frequencies, and differ somewhat for the high frequencies.

These relations reflect the observations already made that the middle ear itself introduces only moderate phase changes in the low and intermediate ranges and has larger effects in the high range. We show in figure 3, for another ear, the differences in the phase patterns obtained before and after removing the middle ear structures. This curve is similar to ones obtained in our former study of the action of the middle ear, and in general our present results confirm those observations.

Figure 4 shows further results after removal of the middle ear structures, and for two electrode positions—one at the round window and one at the apex of the cochlea. The phase changes recorded from the apex are closely similar to those recorded from the round window for the low frequencies and then show differences

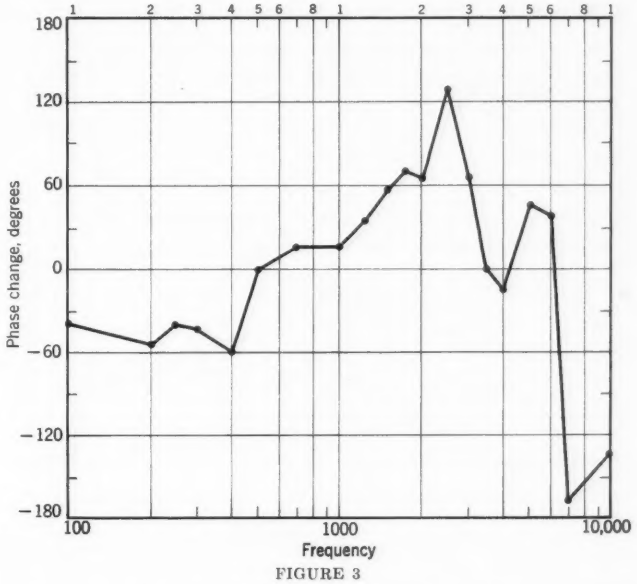


FIGURE 3

Phase changes produced by the middle ear. Shown here are differences between the phase changes recorded when the ear was intact and after the drum membrane and outer ossicles were removed.

as the frequency rises. Beyond about 500 cycles the phase changes recorded from the apex show greater lags than those from the round window, and beyond 1500 cycles the apical curve levels out around 40 to 60 deg. of lag. It will be noted that the apical curve in this figure goes only to 6000 cycles, and in general we were unable to extend our apical observations to the uppermost frequencies. The potentials that may be recorded for the high tones at this position were too small for reliable measurements.

*Discussion.*—In the consideration of these results it should be noted that measurements of phase are subject to certain limitations of interpretation. The usual methods of measuring phase, including the wave-matching method employed here, do not distinguish between a given phase relation and another that differs from it

by 360 deg. or indeed by any integral number of periods. This restriction can be overcome only by measuring the relations between two phenomena in terms of time as such, and these measurements are difficult to achieve with pure tones. We must bear in mind that the absolute phase relations between stimulus and response in these experiments may differ from the relative readings as reported.

Also we must recognize a limitation in the relation between phase and time. Though phase may be regarded as a particular sort of time function, it is not always possible to translate it into time in a meaningful way. To measure a time interval between two events the events themselves must have some feature of occurrence in time that can be taken as significant for the purpose at hand. The moment of initial appearance is such a feature in common use, and the moment of cessation will serve equally well. Two continuous waves lack these features. With such

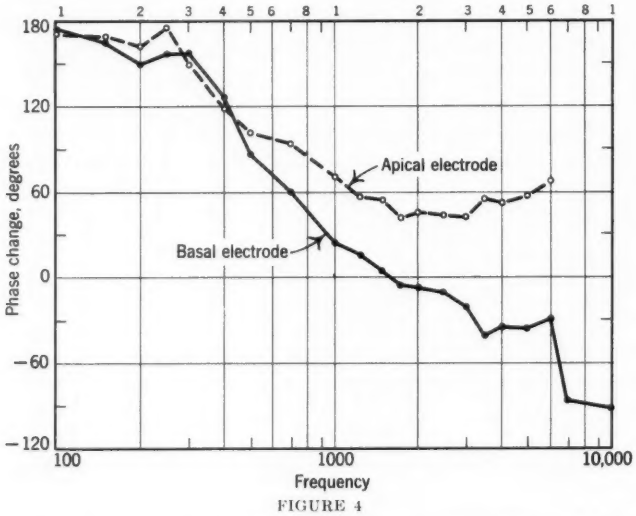


FIGURE 4

Phase changes produced by the inner ear, as recorded from two electrode positions, one at the basal end of the cochlea and another at the apical end.

waves we can relate the peaks occurring in each, or the two troughs, or any other corresponding phase positions, but this procedure runs into the uncertainty just mentioned in that ordinarily we do not know what particular peaks (or other features) in the two wave series are to be related. Our times therefore are indeterminate by any positive or negative number of periods.

It must further be noted that when we are dealing with wave transmission along a conducting system the measurement of phase relations at two points in the system does not usually disclose the time delay of the transmission process—which is the time required for energy to be propagated from molecule to molecule through the system. A measurement in terms of time as such is likewise handicapped when phase changes are present and the times of transmission are short relative to the periods of the waves, as we find to be true for the ear.

In spite of the limitations just mentioned it is still of interest to take the data of these experiments at their face value and to convert the phase changes into time differences. This has been done for the averages of three series of readings like those of figure 4, and the results are shown in figure 5. Here, as before, the solid curve represents measurements with a round window electrode and the dashed curve represents those with an apical electrode. As indicated, the 100-cycle tone suffers a "delay" of nearly 5 milliseconds, and then this "delay" rapidly diminishes as the frequency rises. Beyond about 1000 cycles the curves run with remarkable uniformity in the region of "no delay." The differences from zero are significant even though they are small. For the apical function the values in the high-tone region are consistently above the zero line and for the basal function they are below this line.

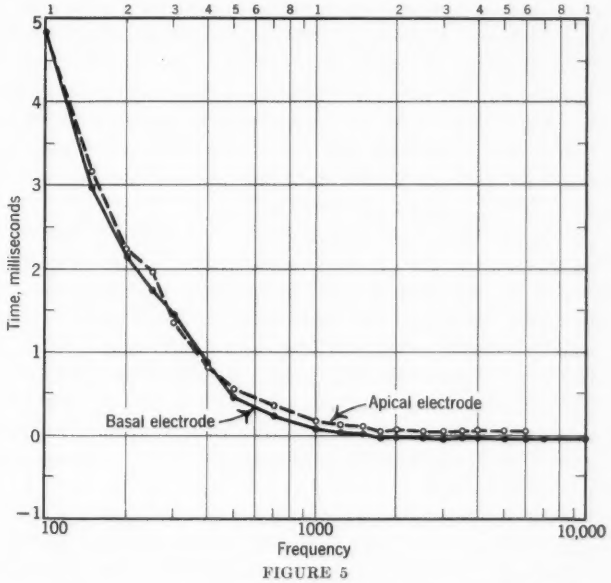


FIGURE 5

Phase changes as in the preceding figure when translated into time. Positive time values correspond to phase delays.

In this connection it may be mentioned that some investigators have attempted to measure the latency of the cochlear potentials, but without recognizing the limitations imposed by the shortness of the paths of transmission and by the presence of phase changes, as discussed above. Davis, Derbyshire, Lurie, and Saul[6] produced clicks by applying condenser discharges to a loudspeaker and sought to measure the time difference between the electrical output of a condenser microphone picking up the sound and the cochlear response of the ear. For this purpose they observed the two waves on the face of a cathode-ray oscillograph. They attempted to relate such features as moment of incidence and the appearance of the more prominent spikes of the complex train of waves, and came out with a latency of 0.1 millisecond. To the theoretical difficulties facing this procedure there are these also:

the two trains of waves were of different form so that corresponding parts could hardly be identified, and the investigators took no account of responsive characteristics of their condenser microphone and amplifying equipment. Accordingly, the latency figure just mentioned is not to be taken seriously as a quantitative measurement, though probably it is of the proper order of magnitude.

Let us now consider the theoretical implications of our results. From earlier studies of the cat's middle ear we already know something of the composition of this more peripheral portion of the mechanical system.[7] It consists of at least four masses, three stiffnesses, and two frictional elements so proportioned and interconnected as to provide two principal resonances and one antiresonance within the ear's working range. The result of this arrangement is the provision of high sensitivity over a broad region of frequencies. The phase changes introduced by this system are only moderate except at the upper end of the frequency scale.

The present experiments show that further phase changes appear in the action of the inner ear, especially in response to the low tones. At this time we are unable to enter into an analysis of the inner ear mechanics to the extent that we have done for the middle ear, because our phase data represent the combined performance of the mechanical system of the cochlea and of the electrical processes by which the cochlear potentials are generated. Without further experimentation it is not possible to separate these two stages of cochlear action.

However, the simple form of the phase pattern as revealed in this total inner ear activity allows us to make certain general inferences. This form of the pattern signifies that both the mechanical and the electrical processes are simple. The mechanical system therefore must be made up of but a few reactive elements; and possibly it consists of one mass, one stiffness, and a moderate amount of frictional resistance. It is not necessary that the magnitudes of these elements be constant throughout the frequency range, and indeed most theories of cochlear mechanics require that they vary. Our results show that if these magnitudes vary they do so in a regular and progressive manner as a function of frequency, as is usually assumed also for theoretical purposes.

Theoretically, the differences in phase or time as shown in figures 4 and 5 between the responses recorded at basal and apical ends of the cochlea might be accounted for in any of three ways—in terms of acoustic conduction in the cochlea, in terms of the response properties of the sensory structures, and in terms of electrical conduction from the responsive structures to the electrodes. The third of these ways we dismiss at once as negligible, because electrical conduction is exceedingly rapid and earlier studies have shown that the electrical path from base to apex of the cochlea is a resistive one.[7] We shall now consider the two remaining possibilities.

1. Sounds applied to the stapes are communicated to the cochlear fluid at the basal end of the cochlea and travel through this fluid to the apical end. The vibrations in the apical region are delayed relative to those in the basal region by an amount that depends upon the distance and the speed of conduction. In the cat the mean fluid pathway from base to apex is about 23 per cent shorter than the basilar membrane, or about 17.4 mm. long on the average. If the speed of sound in the cochlear fluid is of the order of magnitude of that of sea water, or about 1400 meters per second according to some of our calculations,[7] the time delay from base to apex will be about 12 microseconds.

It should be mentioned, however, that certain auditory theories, known as the traveling wave theories, have assumed that sounds applied at the basal end of the cochlea travel to the apical end by a wave motion along the basilar membrane and its related structures. This form of conduction through the cochlea is regarded as relatively slow, with estimates of its speed ranging from 5 cm. per second to 790 meters per second. In most of the wave theories the speed is said to vary considerably with frequency, but for purposes of discussion we can take a figure of 30 meters per second as reasonably representative of the range. A speed of 30 meters per second would give a time delay along the cat's basilar membrane (which is 22.5 mm. long on the average) of about 780 microseconds.

The delay in sound conduction from base to apex would be fully reflected in the responses measured by our two electrodes only if stimulation occurred all along the cochlea and each electrode picked up potentials exclusively from its own region. Earlier experiments have shown that any tonal stimulus does indeed affect the sensory structures throughout the cochlea, though in varying degree depending on the frequency so that for each tone there is a typical pattern of activity. An electrode most readily picks up the potentials generated in its immediate vicinity, but it records also from all other regions to a degree depending upon the resistance of the intervening fluids and tissues. Our measurements show that the fall in potential due to this resistance is about 1 db. per mm. of distance. The result is that electrodes at the ends of the cochlea give different recordings of any given pattern of activity, except for the particular situation in which this pattern is uniform along the cochlea or is symmetrically disposed between the two ends. It is of interest to calculate the locations of the centers of the effective patterns—or of the electrical fields as seen by our electrodes—but to do so it is necessary to know the form of the action pattern. For the simple condition of uniform stimulation, which probably is approximated for the very low tones, these centers are separated by about a third of the length of the cochlea, or by distances of 5.8 or 8.5 mm. depending on whether fluid or membrane transmission is assumed. An earlier calculation for 10,000 cycles, based on a pattern of activity derived from studies of maximum cochlear responses and stimulation deafness experiments, has indicated an effective separation of the centers of the fields tapped by basal and apical electrodes of 6.8 mm. under the assumption of fluid transmission and 8.9 mm. under the assumption of membrane transmission. At the suggested speed of sound in cochlear fluid (1400 meters per second) these distances will be traversed in 5 microseconds or less, and at the speed indicated for membrane transmission (30 meters per second) in about 300 microseconds.

The results of figures 4 and 5 show no consistent differences for the low tones. These results do not deny the possibility of slight differences of a few degrees of phase or a few microseconds of time, because such differences might easily have been obscured by errors of measurement. Larger differences of the order of 300 microseconds, however, can hardly have escaped notice. These results therefore are consistent with our estimations of delay times through the cochlea of something like 5 microseconds, but are not consistent with the longer times required by the traveling wave theories. For the high tones our results show phase differences of 57 to 85 deg. or average time delays of 45 microseconds. These times are longer than we have estimated for sound conduction alone (i.e., 5 microseconds) and we believe

that the excess is to be ascribed to the responsive characteristics of the cochlear structures, now to be discussed.

2. The responsive structures of the cochlea have inertia, stiffness, and frictional resistance, and these properties vary to some extent from one end of the cochlea to the other. From base to apex the mass of the structures lying on the basilar membrane increases three to five fold and the stiffness, according to Békésy's measurements, diminishes by 100-fold. If these structures are not perfectly coupled to the cochlear fluid from which sound vibrations reach them they will respond somewhat independently of the fluid and of one another, and one aspect of this variation is the phase or time delay of their motions. It is possible that further delay appears in the production of electrical potentials, but our guess is that this production is rapid and reasonably uniform among the cochlear structures and that our results as shown in fig. 2 are mainly to be explained by differences in the mechanical responses. The differences to be seen in figs. 4 and 5 between basal and apical recording likewise, we believe, are to be accounted for principally by the mechanical differences as made effective at our two electrode positions.

The greater phase delay recorded at the apical position for the high tones is to be expected in view of the structural differentiation along the basilar membrane: the more apical structures, with relatively large mass and small stiffness, will exhibit the greater phase lags. For the lowest tones a systematic phase difference is not distinguishable in the responses from the two ends of the cochlea, evidently because these tones spread broadly in their actions on the basilar membrane and have response functions that are recorded with about equal effectiveness at the two electrode positions.

*Summary.*—Experiments were carried out on the cat to study the phase relations between the pressure waves of sound stimulating the ear and the resulting waves of electrical potential in the cochlea. Measurements were made under two conditions, with the middle ear apparatus intact and after removal of the drum membrane, malleus, and incus. The results reveal a systematic pattern of phase changes in the ear as a function of frequency. A large phase lag is found for the low tones, and this lag becomes progressively smaller as the frequency is raised. For the middle and high tones the phase relation is close to zero; for tones above 1500 cycles it is on the positive or lagging side of zero for apical recording and on the negative or leading side for basal recording—which means that for these frequencies the peak of the cochlear potential wave is nearly (but not exactly) coincident with the pressure peak of the stimulating wave. The results are discussed in relation to auditory theory.

* From the Princeton Psychological Laboratory. This research was supported in part by the Office of Naval Research, under Contract N6-onr-270, T.O. 3, Project NR-140-322, and also by Higgins funds allotted to Princeton University.

[1] Wever, E. G., and Lawrence, M., *Ann. Otol., Rhinol., Laryngol.*, St. Louis, **59**, 5–18 (1950).

[2] Wever, E. G., and Lawrence, M., *Ibid.*, **59**, 322–330 (1950).

[3] We are indebted to Dr. F. F. Romanow of the Bell Telephone Laboratories for these determinations.

[4] Wiener, F. M., *J. Acoust. Soc. Am.*, **13**, 115–123 (1941).

[5] Wiener, F. M., *Ibid.*, **20**, 707 (1948).

[6] Davis, H., Derbyshire, A. J., Lurie, M. H , and Saul, L. J., *Am. J. Physiol.*, **107**, 311–332 (1934).

[7] Wever, E. G., and Lawrence, M., *Physiological Acoustics*, 1954.

# *CONFERENCE ON BIOCHEMISTRY, PALEOECOLOGY, AND EVOLUTION*

By W. P. Woodring

U. S. GEOLOGICAL SURVEY, WASHINGTON, D. C.

*Communicated January 11, 1954*

*Introduction.*—Progress in biochemistry during the last few decades has opened new avenues for advances in geology, paleoecology, paleontology, and the study of evolution. To catalyze progress in exploiting these possibilities, an informal Conference on Biochemistry, Paleoecology, and Evolution was held at Shelter Island, Long Island, June 9–11, 1953, under the auspices of the Academy. Twenty-three biochemists, biophysicists, biologists, paleontologists, geologists, and geochemists participated (P. H. Abelson, E. S. Barghoorn, W. Bergmann, H. F. Blum, W. H. Bucher, P. E. Cloud, Jr., Carl Epling, Bently Glass, S. Granick, S. B. Hendricks, G. E. Hutchinson, G. L. Jepsen, J. L. Kulp, Bernhard Kummel, H. S. Ladd, H. A. Lowenstam, W. D. McElroy, D. A. MacInnes, B. H. Mason, A. S. Romer, W. W. Rubey, G. G. Simpson, W. P. Woodring). Eight sessions were held under the direction of Abelson, Cloud, Epling, Hutchinson, McElroy, Mason, Rubey, and Simpson as discussion leaders. The following brief report is based principally on summaries prepared by Glass, Granick, Kulp, and Ladd, who served as reporters, and on editorial advice from Abelson, Cloud, and Rubey. In this summary account it is impractical to mention numerous contributions, including contributions from participants whose names do not appear. Throughout the sessions, Hutchinson in particular guided the discussion into profitable channels. Although it is impossible to convey on paper the spirit of enthusiasm of the meeting, some examples of the topics discussed will serve to indicate the areas which were vigorously examined.

*Biochemistry.*—A modified Oparin-Horowitz-Bernal scheme of the origin of life was outlined by McElroy. He emphasized the possible role of group transfer in the early production of complex molecules. An absorptive surface such as clay could serve as an inorganic catalyst in the absence of enzymes. Thereby phosphate, methyl, acetyl, and peptide could be exchanged without loss of bond energy. Hendricks pointed out that clay would also combine with purines, pyrimidines, and phosphates. Thus several of the components of nucleic acid could get together on clay.

Granick discussed the hypothesis that the fundamental functions of protoplasm, such as oxidation and photosynthesis, may have been carried out by relatively simple compounds during early stages of protoplasm development. As protoplasm evolved, these functions came to be carried out with greater efficiency by more complex compounds. As an example involving oxidation metabolism, he pointed out that the present iron porphyrin proteins all cooperate to bring about oxidation. These iron porphyrin proteins may be classified into five groups according to their specific activities. Inorganic ferrous-ferric iron or its hydroxides, however, possess all these activities, but in low degree. Thus at an early stage the simpler inorganic iron compounds may have functioned in essentially the same manner as later more complex iron compounds, which came to have highly selective and enhanced activities.

As an illustration of the view that selection of a particular system as most useful may follow concomitant development of a number of systems, Granick cited hemoglobin. Though hemoglobin appears in many of the invertebrates, only the vertebrates have used it to great advantage. Its selective advantage, however, depended on the evolution of other systems to satisfy the high iron requirements: the HCl of the stomach to dissolve the iron of food and to make it more readily absorbable; a regulatory mechanism in the intestinal mucosa for absorbing more or less iron as the occasion requires; a transport protein, transferrin, to transport the iron in the blood stream; and an iron storage protein, ferritin, to store the iron in liver, spleen, and bone marrow.

Glass cited the evolution of the mitotic mechanism, so nearly universal and so basically similar, as the best argument for a single origin of life. Only the blue-green algae and possibly the bacteria still survive as representatives of a more primitive organization. The origin of sexual reproduction is equally basic to evolution and almost as primitive.

Hutchinson suggested consideration of the pattern of evolution in the range of biochemical composition. The testing ground for various kinds of metabolism may be found in the invertebrates. As an example of comparative biochemistry, Bergmann discussed his work on sterols. Sponges are exceptionally rich in sterols. They contain little or no cholesterol, but at least 15 other sterols have been found in an examination of 50 species, as many as four in one sponge. In the vertebrates the diversity of sterols is reduced and cholesterol is the typical sterol. Fell recently found that a consistent interpretation of embryological evidence led to the conclusion that echinoids and ophiuroids are closely related, and that asteroids and holothurians are closely related. Fell rejected the results on conventional grounds of adult morphology. His results are substantially supported by biochemical evidence. Cholesterol is the principal sterol of all echinoids so far examined and has been tentatively identified in the only ophiuroid so far studied. On the contrary, stellasterol and the similar hitodesterol are the principal sterols of asteroids, and stellasterol of holothurians.

The phosphagens lend further support to Fell's results. Baldwin has called attention to the distribution of arginine and creatine. Arginine is found in nearly all invertebrates, creatine in all vertebrates. Echinoids and ophiuroids are exceptional among the invertebrates, for both arginine and creatine occur in echinoids, only creatine in ophiuroids.

The distribution of sterols and phosphagens also supports the embryological evidence that the protochordate acorn worms represent a link between the echnioderms and the vertebrates.

Bergmann further pointed out that the fatty acids reveal an evolutionary pattern similar to that of the sterols. Invertebrates have a wide spectrum of fatty acids. Sponges, for example, contain fatty acids ranging from C-14 to C-28, C-24 and C-26 being most abundant. The fatty acids of vertebrates are mostly of C-16 and C-18 structure (stearic, oleic, palmitic).

In summary, Hutchinson developed the view that simpler organisms experimented, so to speak, with a wide range of chemical compounds and more complex forms specialized in a limited range.

*Geochemistry.*—The role of geochemistry in imposing limits on speculations concerning the origin of life and in evaluating paleoecology was emphasized and also the contributions that biochemistry may eventually make to geochemistry. Regardless of the manner in which life originated, its mere persistence since that time furnishes information, not now understood, about the subsequent history of the earth and the living matter thereon. Rubey reviewed some aspects of his studies on the geological history of sea water. The mineral constituents of ancient muds and other sediments suggest strongly that the temperature and chemical composition of the ocean and atmosphere have changed only slightly through much of the geologic past. But if the fossils preserved in these sediments could be interpreted in terms of their biochemistry, we would have far more precise knowledge than that now available about the environmental conditions of the past.

The fundamental life-processes of fertilization, reproduction, respiration, digestion, etc., depend upon the presence and specific catalytic action of particular enzymes; and the activity of these enzymes is closely dependent upon the temperature, chemical composition (including minor as well as major constituents), and concentration of various ions in the surrounding media. Many present-day organisms can tolerate only slight changes in the physical and chemical conditions of their environment (as, for example, the narrow range of pH of sea water within which *Arbacia* eggs are fertilized). Some groups have developed special organs, such as the kidney, which afford partial protection against certain aspects of the external environment; but other groups, such as the echinoids, cephalopods, and brachiopods, appear to have no comparable mechanisms for protection. To what extent may such unprotected organisms adapt themselves to significant changes in their external environment without substituting different enzymes to replace those on which their life-processes have previously depended? And to what extent would such biochemical adaptations be evident in the morphology of the organisms affected particularly in the hard parts available for paleontological study? It will require the collaboration of biochemists, paleontologists, and geochemists to develop this neglected field of investigation.

Mason pointed out that there are good grounds for attributing most of the chlorine in the ocean to volcanoes. What part of the chlorine in volcanic emanations, however, came from the interior of the earth? Kulp presented data on the absence of $C^{14}$ in the $CO_2$ collected at Kilauea, indicating that at least 98 per cent of the $CO_2$ is primary, unless the time constant for the seepage of ocean water into the volcano is greater than 30,000 years.

The geological timetable was reviewed by Mason, who emphasized the increasing precision due to new measurements and new methods. A reliable time scale is a prerequisite for consideration of pre-Cambrian evolution.

*Paleontology and Paleoecology.*—Romer discussed the loss of ossification in several lines of Triassic amphibia—a loss of ossification accompanied in some groups by an increase in dermal armor. Though several suggestions were made concerning possible biochemical control, none seemed to be entirely satisfactory.

Romer cited geological evidence for the view that the earliest fishes lived in fresh water and that at a later time placoderms and shark-like fishes and still later bony fishes invaded the sea. The nitrogen-excreting mechanism of fishes supports this view.

Lowenstam reported on the discovery of a temperature effect on the carbonate deposition of certain marine animals. In serpulid worm tubes aragonite increases over calcite with increasing temperature. The same effect has also been detected in a number of gastropod and pelecypod species. Deviations from the gross trend suggest superposition of lesser effects by other environmental factors, possibly including salinity. Controlled tank experiments are planned to determine the role of the various factors. Data were presented to show that concentration of the trace constituent strontium in serpulid worm tubes rises with the increase in aragonite as the temperature rises and therefore is indirectly sensitive to temperature.

Lowenstam also mentioned the work in collaboration with Epstein on the climatic history of Cretaceous post-Aptian seas, principally of western Europe, as indicated by the $O^{16}/O^{18}$ ratio of the skeletal calcium carbonate of belemnites and some associated fossils. Cyclical temperature variations, based on analyses of cross-sections of single belemnites, are interpreted to indicate latitudinal migration rather than seasonal depth migration.

Woodring raised the question whether physiological differentiation, as shown by habitat and habits, is likely to be correlated with biochemical differentiation. Though the opinion was expressed that different physiological aspects may have the same biochemical background, paleontologists would be interested in the comparative biochemistry of species that are closely related morphologically—or even indistinguishable on the basis of characters available to paleontologists—but have different physiological characters.

Cloud exhibited an alga-like fossil from Upper Cambrian rocks of Texas that shows filamentous branching structure. Though fossils of the same gross form range well back into the pre-Cambrian, the specimen exhibited is, so far as known, the oldest to show such structure. He cited examples of fossil organic material possibly suitable for biochemical studies, including original organic matter from the Pleistocene; organic material in the lacustrine Green River formation of middle Eocene age, the Permian Phosphoria formation, the Devonian-Mississippian black shale suite, the Upper Cambrian Kulm of Sweden, and the Middle Cambrian Burgess shale member of the Stephen formation. A Middle Devonian terebratuloid brachiopod, which was exhibited, has maroon color bands that presumably contain original pigment and should be examined for carotenoids or protoporphyrins. Original shell material probably is preserved in calcaro-phosphatic brachiopods of Cambrian to Silurian age. Pseudokeratin or similar material is preserved in fossils representing several phyla and a wide age range. Even deposits as old as late Paleozoic contain aragonite shells, apparently original shell material protected by enclosing asphalt. Barghoorn pointed out that much material is available among fossil plants, including cellulose as old as Carboniferous, and that porphyrin has been isolated from anthracite coal of Carboniferous age. Bergmann cited Jurassic crinoids that have bright red disks, perhaps due to preservation of a quinoid pigment. The label of a Lower Jurassic squid in the British Museum is written in ink from its own ink sac.

The importance of black shales was emphasized. Lowenstam aptly designated them anerobic burial traps. They contain much unidentified organic matter and are rich in trace elements.

*Suggestions.*—Suggestions for biochemical studies bearing on evolution and

paleoecology were summarized and discussed by Abelson during the closing session.

Evolution in the ordinary sense is an elaboration of pre-Cambrian and possibly very Early Cambrian developments that laid the basic patterns. Anything that might give clues to pre-Cambrian evolution would be significant. Discovery and characterization of pre-Cambrian fossil biochemicals would be particularly valuable. Did the graphite in pre-Cambrian schists originate from the reduction of carbonate or was it of organic origin? Attempts have been made to differentiate organic from inorganic graphite by measurements of the ratio of $C^{12}$ to $C^{13}$. Results suggest that some of the graphite may be of organic origin, but the evidence is not conclusive. A variety of experimental tests could bring forth considerably more evidence on this question. The isotope studies could be expanded to include measurements of $S^{32}/S^{34}$ abundance ratios. The graphite could be examined for the presence of other elements usually associated with living processes, such as nitrogen, phosphorus, and sulphur. The presence of heterocyclic compounds containing nitrogen would be of particular interest. Fluorescence may reveal the presence of organic compounds in pre-Cambrian sedimentary rocks.

A systematic study of the organic and inorganic compounds found in marine black shales would be valuable. Such an investigation on shales of increasing age would provide data on the aging processes of organic compounds in geological settings. Black shales contain a relatively high concentration of certain trace elements. Some organic substances are good ionic-exchange materials and trace elements would tend to accumulate both during the life of the organism and after its death. Knowledge of the specific ensembles of elements concentrated as a result of these processes could aid in the establishment of conclusive evidence regarding past biological events.

The ratio of strontium to calcium, which is much higher in sea water than in fresh water, is a potential tool in paleoecological studies. Study of the biochemistry of calcite and apatite deposition and a study of bone formation in the bony as compared with the cartilaginous fishes would be of value, especially for paleoecology.

Biochemical studies of exceptionally preserved fossils would yield interesting results. The biochemistry of "living fossils"—such as the South African coelacanth fishes, the Port Jackson shark, and the sole surviving rhynchocephalian reptile (the New Zealand tuatara)—may give clues to patterns of biochemical evolution.

Expansion of studies on comparative biochemical processes and biosynthetic chains would provide valuable information on possible evolutionary trends. Additional studies of the biochemistry of embryological development, such as Needham's work on nitrogen excretion in the frog, might be useful in providing data on the origins of biochemical systems and the origins of differentiation.

The hope was expressed that some of the suggestions may be followed up as a part of current or new programs.

### SELECTED BIBLIOGRAPHY

Baldwin, Ernest, *An Introduction to Comparative Biochemistry*, 3rd ed., Cambridge, 1949, 164 pp., 12 figs.; *Dynamic Aspects of Biochemistry*, Cambridge, 1949, 457 pp., 34 figs.

Beadle, L. C., "Osmotic Regulation and the Faunas of Inland Waters," *Biol. Revs., Cambridge Phil. Soc.*, **18**, 172–183 (1943); 3 figs.

Bergmann, Werner, "Comparative Biochemical Studies on the Lipids of Marine Invertebrates, with Special Reference to the Sterols," *J. Marine Research*, **8,** no. 2, 137–176 (1949).

Bernal, J. D., *The Physical Basis of Life*, London, 1951, 80 pp.

Blum, H. E., *Time's Arrow and Evolution*, Princeton, 1951, 222 pp., 20 figs.

Breder, C. M., Jr., "A Consideration of Evolutionary Hypotheses in Reference to the Origin of Life," *N. Y. Zool. Soc., Zoologica*, **27,** no. 19, 131–143 (1942); 1 fig.

Cloud, P. E., Jr., "Some Problems and Patterns of Evolution Exemplified by Fossil Invertebrates," *Evolution*, **2,** 322–350 (1948); 4 pls., 4 figs.

Comfort, Alex, "The Pigmentation of Molluscan Shells," *Biol. Revs., Cambridge Phil. Soc.*, **26,** 285–301 (1951); 5 figs.

Conway, E. J., "Mean Geochemical Data in Relation to Oceanic Evolution," *Proc. Roy. Irish Acad.*, **48,** sect. B, no. 8, 119–159 (1942). "The Chemical Evolution of the Ocean," *Ibid.*, no. 9, 161–212 (1943).

Fell, H. Barraclough, "Echinodern Embryology and the orgin of Chordates," *Biol. Revs., Cambridge Phil. Soc.*, **23,** 81–107 (1948) 8 figs.

Florkin, Marcel, *Biochemical Evolution* (translation by Sergius Morgulis), New York, 1949, 157 pp., 25 figs.

Granick, S., "The Structural and Functional Relationships Between Heme and Chlorophyll," Harvey Lectures, ser. 44, 220–245 (1950); 6 figs.

Henderson, L. J., *The Fitness of the Environment*, New York, 1913, 317 pp.; reprinted 1927.

Horowitz, N. H., "On the Evolution of Biochemical Syntheses," PROC. NATL. ACAD. SCI., **31,** 153–157 (1945).

Hutchinson, G. E., "Conway on the Ocean; an Appreciation and a Criticism," *Am. J. Sci.*, **242,** 272–280 (1944).

Keys, Ancel, "The Mechanism of Adaptation to Varying Salinity in the Common Eel and the General Problem of Osmotic Regulation in Fishes," *Proc. Roy. Soc. (London)*, **112,** ser. B, 184–199 (1933); 8 figs.

Kutscher, F., and Ackermann, D., "Comparative Biochemistry of Vertebrates and Invertebrates," *Ann. Rev. Biochem.*, **2,** 355–376 (1933); **5,** 453–462 (1936).

Lowenstam, H. A., and Epstein, S., "Cretaceous Post-Aptian Paleotemperatures, Chiefly from Western Europe," *J. Geology* (in press).

Needham, Joseph, "The Biochemical Aspect of the Recapitulation Theory," *Biol. Revs. and Biol. Proc., Cambridge Phil. Soc.*, **5,** 142–158 (1930); 1 fig. "Contributions of Chemical Physiology to the Problem of Reversibility in Evolution," *Biol. Revs., Cambridge Philos. Soc.*, **13,** 225–251 (1938); 4 figs.

Needham, Joseph, and Needham, D. M., "Biochemical Evidence Regarding the Origin of Vertebrates," *Science Progr.*, **26,** 626–642 (1932).

Oparin, A. I., *The Origin of Life* (translation by Sergius Morgulis), New York, 1938, 170 pp., 8 figs.

Raymond, P. E., "Pre-Cambrian Life," *Geol. Soc. Am. Bull.*, **46,** 375–392 (1935).

Redfield, A. C., "The Evolution of the Respiratory Function of the Blood," *Quart. Rev. Biol.*, **8,** 31–57 (1933); 5 figs.

Romer, A. S., "Cartilage an Embryonic Adaptation," *Am. Naturalist*, **76,** 394–404 (1942).

Romer, A. S., and Brandon, H. G., "Environment of the Early Vertebrates," *Am. Midland Naturalist*, **16,** 805–856 (1935); 2 figs.

Rubey, W. W., "Geologic History of Sea Water," *Geol. Soc. Am. Bull.*, **62,** 1111–1147 (1951); 4 figs.

Simpson, G. G., *Tempo and Mode in Evolution*, New York, 1944, 237 pp., 36 figs. *The Meaning of Evolution; a Study of the History of Life and of Its Significance for Man*, New Haven, 1949, 364 pp., 38 figs.

Smith, H. W., "Water Regulation and Its Evolution in the Fishes," *Quart. Rev. Biol.*, **7,** 1–26 (1932).

Urey, H. C., Lowenstam, H. A., Epstein, S., and McKinney, C. R., "Measurement of Paleotemperatures of the Upper Cretaceous of England, Denmark, and the Southeastern United States," *Geol. Soc. Am. Bull.*, **62,** 399–416 (1951); 1 pl., 1 fig.

Woodring, W. P., "Basic Assumption Underlying Paleoecology," *Science*, **113,** no. 2939, 482–483 (1951).

Yonge, C. M., "Evolution and Adaptation in the Digestive System of the Metazoa," *Biol. Revs., Cambridge Philos. Soc.*, **12,** 87–115 (1937).

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